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Application of a Glycoproteomics-Based Biomarker Development Method: Alteration in Glycan Structure on Colony Stimulating Factor 1 Receptor as a Possible Glycobiomarker Candidate for Evaluation of Liver Cirrhosis

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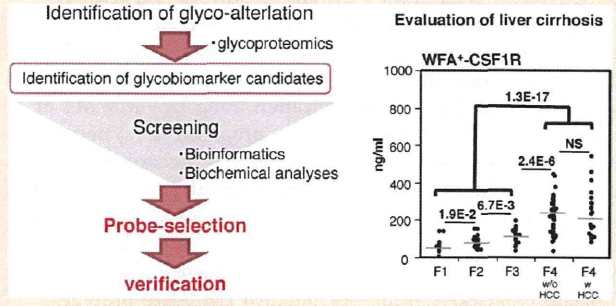
Supporting Information

ABSTRACT: The importance of diagnosis and therapies for liver cirrhosis (LC) is indisputable. Thus, a reliable method for monitoring the progression of liver fibrosis and resultant LC is urgently needed. Previously, using a lectin-assisted glycoproteomic method, we identified 26 serum glycoproteins as promising glycobiomarker candidates for monitoring the progression of liver diseases. In this study, we identified colony stimulating factor 1 receptor (CSF1R) as a promising LC marker candidate and then established *Wisteria floribunda* agglutinin (WFA)-reactive CSF1R (WFA$^+$-CSF1R) as a novel possible glycobiomarker candidate by utilizing a glycoproteomics-based strategy. The serum level of WFA$^+$-CSF1R in patients with hepatitis C virus (HCV)-infected liver disease was measured by an antibody-lectin sandwich ELISA. In a proof-of-concept experiment of the strategy preceding to future clinical studies, LC patients showed a high serum WFA$^+$-CSF1R level in selected samples ($P = 1.3 \times 10^{-17}$). This result suggests WFA$^+$-CSF1R is a possible biomarker candidate for evaluation of LC. Our results verified feasibility of this strategy for glycobiomarker development.



KEYWORDS: *glycobiomarker, glycoprotein, glycoproteomics, liver cirrhosis, colony stimulating factor 1 receptor, lectin array, glycan alteration*

INTRODUCTION

Glycosylation plays an important role in regulating the properties of proteins and lipids on the cell surface. Glycan profiles change significantly along with the cell-autonomous changes or the changes in the pericellular surroundings. Cancer-related structural modifications of glycans, such as sialylation, multiple branching polylactosamine elongation and fucosylation are caused by cellular malignant transformation.[1−4] Additionally, expression levels of sialyl Lewis x antigens are elevated in a hypoxic environment.[5] Many test kits used for clinical diagnosis of cancer, such as the kits for the tumor markers CA19-9 and CA125, have been developed based on the observed alterations in the glycan structures. Currently, certain types of cancers are diagnosed by measuring levels of specific serum proteins. However, sometimes these serum proteins are secreted from the background lesions of inflammatory diseases associated with cancer or from other physiologically associated organs.[6] This observation suggests that these serum proteins are not good biomarkers for cancer diagnosis as their increased levels might not always reflect the cancer itself. Therefore, to find a disease-specific biomarker, it would be more advantageous to monitor alterations in the glycan structures as an indicator of the disease status rather than measuring the quantitative changes in the protein. Indeed, several serum glycobiomarkers have been developed as possible diagnostic or prognostic indices for liver diseases. For example, the *Lens culinaris* agglutinin (LCA)-reactive α-fetoprotein (AFP) AFP-L3 is a more specific biomarker for the diagnosis of hepatocellular carcinoma (HCC) than the AFP.[7] Similarly, *Aleuria aurantia* lectin (AAL)-reactive serum proteins (e.g., complement component 3) have been proposed as the diagnostic marker for early HCC,[8] and a multilectin-based assay for the serum α1-acid glycoprotein (AGP) has been

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found to be useful for specific evaluation of fibrosis stage.[9,10] All of these glycobiomarkers contribute to improved diagnostic specificity.

It is estimated that worldwide about 350 million people are infected with chronic hepatitis B virus (HBV) and over 170 million people are infected with hepatitis C virus (HCV).[11] Approximately 20–30% of patients with chronic HCV infection develop liver cirrhosis (LC) over a period of 20 years. Although a considerable proportion of the patients eventually progress to the decompensated LC stage over a longer period, some of the patients remain at the compensated stage for a long time.[12] Once LC has developed, complications including gastrointestinal bleeding, portosystemic encephalopathy, and ascites are common. In Japan, about 2 million people are HCV carriers and 1.5 million people are HBV carriers, out of which 40,000 people per annum die of LC and HCC, and 10,000 people per annum die of LC-related complications. Patients with HCV-related compensated LC often progress to the decompensated LC stage,[13] and HCC occurs in 8% of LC patients per annum. Therefore, reliable methods or markers are needed for the evaluation of LC progression and its subsequent therapy.

Recently, attempts were made to comprehensively identify serum glycobiomarkers by using a combination of proteomic and glycomic techniques.[8] Several published reports have described methods for identification of candidate biomarkers for the liver disease, including those that used fucose-binding lectins as a glycan probe to screen for altered fucosylated proteins followed by their identification using LC–MS/MS or antibody arrays.[8,14] Previously, we proposed a systematic strategy for developing glycobiomarkers.[15] Accordingly, we identified candidate biomarkers for liver disease using the fucose-binding lectin AAL followed by a LC–MS/MS-based technique with isotope-coded glycosylation site-specific tagging (IGOT-LC–MS) and subsequently identified 26 promising candidate biomarkers from 744 putative candidate proteins.[16] However, in this previous study, verification was performed by comparative analysis of samples collected from healthy volunteers (HV) and patients with LC complicated by HCC as a state of progressed liver disease. Accordingly, these candidates included biomarker candidates for various stages of liver disease (i.e., biomarker candidates for liver fibrosis, LC, and HCC), and we should determine which biomarker candidate would identify which stage of chronic hepatitis and whether the onset of HCC affected their serum levels.

In the present study, we selected a new marker for LC from 26 previously identified candidate marker proteins[16] and determined its characteristics. As a result, we effectively identified glycoprotein colony stimulating factor 1 receptor (CSF1R) as the most promising candidate LC marker. We also performed antibody-assisted lectin profiling (ALP) analysis to select a suitable glycan probe, *Wisteria floribunda* agglutinin (WFA), to construct an antibody-lectin sandwich enzyme-linked immunosorbent assay (ELISA) system for use in a number of samples. Furthermore, we demonstrated a correlation between the serum level of WFA-reactive CSF1R with progression level of LC.

MATERIALS AND METHODS

Specimens

This study was approved by the ethical committee of the Nagoya City University Hospital and National Institute of Advanced Industrial Science and Technology. All participants gave their written informed consent. Pooled serum samples for ALP analysis were prepared by mixing equal amounts of serum obtained from subjects as follows: pool-1 is a mixture of 5 HV individuals (HV01–HV05), pool-2 is a mixture of 14 HV individuals (HV06–HV19), pool-3 is a mixture of 5 LC with HCC patients (LC01–LC05), and pool-4 is a mixture of 4 LC with HCC patients (LC06–LC09) (Table 1). Clinical information on participants is summarized in Table 2. Liver fibrosis was diagnosed by biopsy, and the stage was assigned according to the new Inuyama classification.[17]

Table 1. Clinical Information on Participants Whose Sera Samples Were Used in the Lectin Microarray Analysis[a]

sample ID	virus infected	status of liver	sex	age
		pool-1		
HV01	NI	healthy	M	70
HV02	NI	healthy	F	73
HV03	NI	healthy	F	53
HV04	NI	healthy	M	37
HV05	NI	healthy	M	68
		pool-2		
HV06	NI	healthy	M	29
HV07	NI	healthy	M	40
HV08	NI	healthy	M	31
HV09	NI	healthy	F	37
HV10	NI	healthy	M	34
HV11	NI	healthy	M	37
HV12	NI	healthy	F	32
HV13	NI	healthy	F	29
HV14	NI	healthy	F	28
HV15	NI	healthy	M	34
HV16	NI	healthy	F	48
HV17	NI	healthy	F	33
HV18	NI	healthy	M	31
HV19	NI	healthy	M	29
		pool-3		
LC01	HCV	LC with HCC	F	77
LC02	HCV	LC with HCC	F	71
LC03	HCV	LC with HCC	F	80
LC04	HCV	LC with HCC	M	70
LC05	HCV	LC with HCC	M	76
		pool-4		
LC06	HBV	LC with HCC	M	55
LC07	HCV	LC with HCC	M	74
LC08	HBV	LC with HCC	M	65
LC09	HBV	LC with HCC	F	60

[a]Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; F, female; M, male; NI, not infected.

Analysis of Microarray Data

Gene expression data available in the public domain were downloaded from the Gene Expression Omnibus (GEO, GSE 25097; www.ncbi.nlm.nih.gov/geo/). The GSE250907 database contains DNA microarray gene expression data of the following samples: HV (n = 6), LC (n = 40), NT (nontumor; n = 243) and HCC (n = 268). All raw data were normalized using Robust Multichip Average (RMA). The data matrix was generated from the gene expression data of 24 promising candidates (out of 26 candidates included in the group I of a previous study[16]). Finally, the correlation analysis was performed.

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Table 2. Basic Information on Patients Whose Sera Samples Were Used in ELISA[a]

	group				
	F1	F2	F3	F4 without HCC	F4 with HCC
fibrosis stage	F1	F2	F3	F4	F4
HCC (+)/(−)	(−)	(−)	(−)	(−)	(+)
no. of patients	26	27	20	46	22
age, mean (SD)	49.5 (13.8)	53.0 (12.2)	58.4 (9.1)	66.3 (9.4)	65.7 (10.6)
gender, male/female	11/15	11/16	5/15	23/23	15/7
AST [IU/mL] median (range)	25.0 (14.0−534)	34.0 (17.0−159)	60.0 (33.0−114)	53.5 (19.0−196)	56.5 (27.0−155)
ALT [IU/mL] median (range)	29.5 (11.0−820)	39.0 (17.0−159)	86.0 (25.0−136)	41.5 (12.0−260)	56.5 (17.0−166)
platelets [$\times 10^4$/mm^3] median (range)	20.3 (7.50−29.8)	16.9 (10.3−29.1)	14.3 (10.4−26.2)	8.70 (3.30−18.3)	9.70 (2.90−24.1)
total CSF1R [ng/mL] median (range)	231 (59.2−608)	269 (82.7−649)	349 (81.9−668)	437 (213−811)	383 (88.7−721)
WFA$^+$-CSF1R [ng/mL] median (range)	57.9 (5.00−142.3)	70.7 (41.3−154)	110 (35.5−199)	220 (34.3−553)	235 (85.4−544)

[a]Abbreviations: HCC, hepatocellular carcinoma; SD, standard deviation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CSF1R, macrophage colony stimulating factor receptor; (+), positive; (−), negative. Fibrosis stages were determined according to the new Inuyama classification.

Western Blot Analysis

Samples were electrophoresed under reducing conditions on 10% SDS polyacrylamide gel (DRC, Inc., Tokyo, Japan) and transferred to Immuno-Blot PVDF membranes (BioRad Laboratories, Hercules, CA). The membranes were blocked with 5% Difco skim milk (BD Bioscience, Franklin Lakes, NJ) in PBS containing 0.1% Tween-20 and incubated with 0.1 μg/mL of a biotinylated anti-human CSF1R goat polyclonal antibody (BAF329, R&D Systems, Minneapolis, MN) and then with horseradish peroxidase-conjugated streptavidin 1/3000 diluted with PBS containing 0.1% Tween-20). The membranes were then treated with the Western Lightning chemiluminescence reagent (PerkinElmer, Waltham, MA).

Immunoprecipitation and Antibody-Assisted Lectin Microarray

Pooled serum samples were diluted by adding 0.1% SDS in PBS (pH 7.4) and denatured at 98 °C for 5 min. CSF1R was then immunoprecipitated from the denatured solution using anti-human CSF1R mouse monoclonal antibody (MAB3292, R&D Systems). ALP analyses were performed following the modified method of Kuno et al.[18] To visualize the glycan profiles of CSF1R, interactions of CSF1R with lectins immobilized on the glass slides were detected by the Cy3-streptavidin method. Briefly, the immunoprecipitated protein was released and applied in triplicate to spots of 43 lectins immobilized on glass slides. The glass slides were scanned using an evanescent-field fluorescence scanner (GlycoStaton, GlycoTechnica, Yokohama, Japan).

Sandwich ELISA

We performed the antibody-antibody sandwich ELISA (Ab-Ab ELISA) for CSF1R (total CSF1R) and antibody-WFA sandwich ELISA (Ab-WFA ELISA) for WFA-reactive CSF1R (WFA$^+$-CSF1R) as follows. The antibody (mouse anti-human CSF1R, clone 61715, MAB3292, R&D Systems) was diluted respectively to 1 μg/mL for Ab-Ab ELISA and 4 μg/mL for Ab-WFA ELISA with TBS buffer (pH 8.0). The antibody solution (100 μL/well) was added into each well of the Maxisorp immunoplate (Thermo Scientific, NUNC, 449824, Roskilde, Denmark), the plate was kept for 6 h at room temperature, and finally, the plate was washed with the wash buffer (TBS buffer containing 0.1% Tween-20 and 0.1% NaN_3, pH 8.0). The plate was blocked with the blocking buffer (TBS buffer containing 3% BSA and 0.1% NaN_3, pH 8.0) overnight at 4 °C. For analysis, 1 μL (for Ab-Ab ELISA) or 5 μL (for Ab-WFA ELISA) of the sera was diluted with, respectively, 99 or 95 μL of the blocking buffer. Subsequently, the diluted serum samples were placed in the wells of the plate and incubated at 37 °C for 2 h, and then the wells were washed 8 times with the wash buffer. Biotin-conjugated antibody (goat polyclonal anti-human CSF1R, BAF329, R&D Systems) and biotin-conjugated WFA (Vector Laboratories, Burlingame, CA) were used as the detection probes for Ab-Ab ELISA and Ab-WFA ELISA, respectively. Next, a 1/50,000 diluted HRP-conjugated streptavidin solution (100 μL/well) was added to each well and incubated 1 h at room temperature, and then the plate was washed 6 times with the wash buffer. The substrate solution (100 μL, Thermo Fisher Scientific, Fremont, CA) was then added to each well. Finally, the reaction was stopped by adding 50 μL of 1 M H_2SO_4 solution to each well.

Statistical Analysis

As the levels of the CSF1R protein in the sera showed normal distribution, data obtained for CSF1R were analyzed by Student's *t* test. On the other hand, data obtained for WFA$^+$-CSF1R, levels of which showed non-normal distribution, were analyzed by Mann−Whitney *U* test. JMP ver. 10.0.2 (SAS Institute Japan Ltd., Tokyo, Japan) software was used for the statistical analysis.

Histochemical Analysis for CSF1R and WFA Epitope Expression in LC Liver

For histological studies, tissues were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin wax according to standard procedures. Embedded sections were cut into 2-μm thin slices and were deparaffinized, hydrated, and rinsed in distilled water. The epitopes were recovered by boiling the slides in 100 mM citrate buffer (pH 9.0) for 5 min in a microwave oven. The same sections were treated with blocking buffer for 20 min at room temperature, treated simultaneously with a primary antibody and lectin, and stained separately with a secondary antibody, streptavidin, and dye as follows: anti-CSF1R primary antibody (1 μg/mL clone C20, Santa Cruz Biotechnology, CA) in combination with anti-rabbit IgG-Alexa Fluor 546 secondary antibody (10 μg/mL, Life Technologies, Carlsbad, CA), biotinylated WFA (20 μg/mL, Vector Laboratories) in combination with Alexa Fluor 488-streptavidin (20 μg/mL, Life Technologies) and Hoechst 333242 (Life Technologies). The antibodies and lectin were diluted in the blocking buffer (0.2% Tween-20, 5% glycerol, and 3% BSA in PBS). Antigen was localized by examining the stained sections

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under a fluorescence microscope (BZ-8000, KEYENCE, Yokohama, Japan). Double-fluorescence staining was performed on 12 tissue specimens.

RESULTS

Selection of Target Molecules for the Development of Biomarker Candidate for LC

The strategy used in this study was based on the strategy for developing a glycobiomarker proposed by Narimatsu et al. (Supplementary Figure 1). The flowchart shown in Figure 1 schematically describes the strategy used in this study for developing a glycobiomarker. Previously, we identified 744 biomarker candidates for monitoring liver disease, and from these we then selected 26 promising candidates (Figure 2A).[16] To rapidly find a novel serum biomarker candidate for LC, we next performed a correlation analysis using the DNA microarray gene expression data obtained from GEO (GSE 25097). We first explored the expression of 24 genes in the test samples and compared their levels to the expression level of neuropilin 1 (NPR1) gene (Figure 2B), because NPR1 is known to be overexpressed in the LC liver and is considered a promoter of LC.[19] We excluded C4A and ORM2 from 26 candidate genes, because expression data for these two genes were not available in the GSE 25097 database. As shown in Figure 2C, the correlation factor for CSF1R expression (R = 0.59) is remarkably higher than those for the other 23 genes. It has been reported earlier that there is a strong correlation between the expression level of CSF1R in the liver tissues from the peritumoral region and the risk of mortality.[20] CSF1R is a highly glycosylated receptor glycoprotein whose activation promotes differentiation and proliferation of monocytic cells and some other types of cells including tumor cells.[21] The ectodomain of CSF1R is readily detected in the serum as this domain is cleaved off by intramembranous proteases via a protein kinase-C (PKC)-mediated mechanism.[22] Availability of such biological and biochemical information on CSF1R supports the idea that it could potentially be a serum biomarker candidate for LC. For these reasons, in the present study we focused on CSF1R as the target molecule.

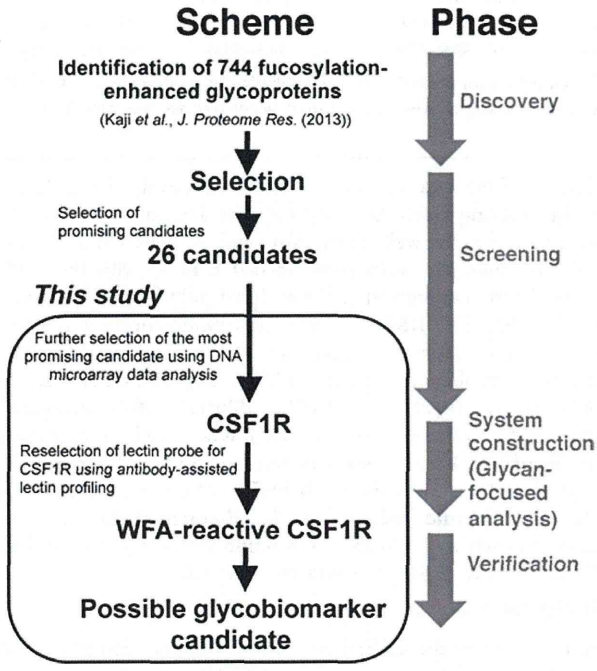

Figure 1. Schematic flow diagram outlining the strategy for glycobiomarker development. We previously identified 744 glycobiomarker candidates, and from these putative candidates we selected 26 serum glycoproteins as the promising candidates (discovery phase[23]). In this study, we screened these 26 proteins and identified CSF1R as the most promising and a novel biomarker candidate for LC (screening phase). Subsequently, we selected WFA as the best lectin for sandwich ELISA assay and constructed a detection system for serum WFA-reactive CSF1R (WFA$^+$-CSF1R) (system construction phase). Finally, we verified the novel possible glycobiomarker candidate using appropriate samples (verification phase).

Selection of Best Lectin for Detecting Liver Disease-Associated Glycan Epitopes of CSF1R

At first, we focused on establishing a lectin-based ELISA for detecting a glycoform of CSF1R whose level would increase with the progression of the liver disease. We previously reported that the AAL-binding affinity of CSF1R from the sera of LC with HCC patients is higher than that from the sera of HV.[16] However, AAL is not an appropriate probe for this system, because AAL binds not only to fucosylated CSF1R but also to other major contaminant glycoproteins including immunoglobulin, which competitively inhibits the specific binding between AAL and fucosylated CSF1R.[23] It was recently reported that, besides increase in fucosylation, other types of glycoform modifications of proteins do occur with the progression of liver fibrosis associated with the abnormality in the glycan formation mechanism.[9] Thus, to circumvent the increased background noise of fucose-binding lectins in sandwich ELISA, one could instead use a lectin that selectively binds to a non-fucose epitope.

We therefore searched for an appropriate lectin that would recognize aberrant non-fucose glycan epitopes on the disease-specific CSF1R glycoforms by performing an ALP analysis of the serum CSF1R. For this purpose, we first enriched CSF1R from the combined serum samples of pool-1 (HV) and pool-3 (LC with HCC) by immunoprecipitation and subjected it to Western blot analysis. As shown in Figure 3A, CSF1R appeared as a protein band of 100−110 kDa. Subsequently, we analyzed glycan profiles of quantitative equivalents of CSF1R by using the ALP analysis. As shown, the signal intensities for AAL and AOL, both of which bind to fucosylated glycans, increased remarkably with the CSF1R that was derived from the sera of pool-3 and pool-4 (LC with HCC) compared to that derived from the sera of pool-1 and pool-2 (HV) (Figure 3B; left panel). Signal intensities for many other lectins also increased with CSF1R derived from the sera of LC with HCC patients (Figure 3B). Three lectins, HHL, ABA, and WFA (Figure 3B, right panel), were specific lectins for LC with HCC. Among these three lectins, WFA showed highest signal intensity. Thus, we selected WFA as the best lectin for detecting liver disease-associated glycan epitopes of CSF1R.

WFA-Reactive CSF1R Is Correlated with Progression Level of LC

Since the relationship between the progression of chronic liver disease and levels of total CSF1R as well as that of WFA$^+$-CSF1R in the serum has not been reported, we developed two ELISA-based assays to quantify total CSF1R and WFA$^+$-CSF1R in the serum (Figure 4A and B). The reliability of Ab-WFA ELISA is shown in Supplementary Figure 2. To determine the

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A

Gene ID	Gene symbol	Description of gene	Expression data in GSE25097 (GEO database)
21071030	A1BG	alpha 1B-glycoprotein	available
4501989	AFP	alpha-fetoprotein precursor	available
122937470	CPN2	carboxypeptidase N, polypeptide 2, 83kD	available
40217843	COMP	cartilage oligomeric matrix protein precursor	available
105990535	F5	coagulation factor V precursor	available
110611237	F13B	coagulation factor XIII B subunit precursor	available
27262659	CSF1R	colony stimulating factor 1 receptor precursor	available
66347875	C1R	complement component 1, r subcomponent	available
4502503	C4BPA	complement component 4 binding protein, alpha chain precursor	available
62912466	C4BPB	complement component 4 binding protein, beta chain isoform 2 precursor	available
67190748	C4A	complement component 4A preproprotein	not available
67782358	CFB	complement factor B preproprotein	available
70906435	FGB	fibrinogen, beta chain preproprotein	available
70906439	FGG	fibrinogen, gamma chain isoform gamma-B precursor	available
47132547	FN1	fibronectin 1 isoform 7 preproprotein	available
29550850	GOLM1	golgi phosphoprotein 2	available
4504489	HRG	histidine-rich glycoprotein precursor	available
70778918	ITIH2	inter-alpha globulin inhibitor H2 polypeptide	available
7669503	LAMP2	lysosomal-associated membrane protein 2 precursor	available
21264359	MASP1	mannan-binding lectin serine protease 1 isoform 2 precursor	available
66864913	NRP1	neuropilin 1 isoform c	available
4505529	ORM2	orosomucoid 2	not available
21361845	PGLYRP2	peptidoglycan recognition protein 2 precursor	available
126273569	CPB2	plasma carboxypeptidase B2	available
4507377	SERPINA7	serine (or cysteine) proteinase inhibitor, clade A, member 7	available
7382460	SHBG	sex hormone-binding globulin	available

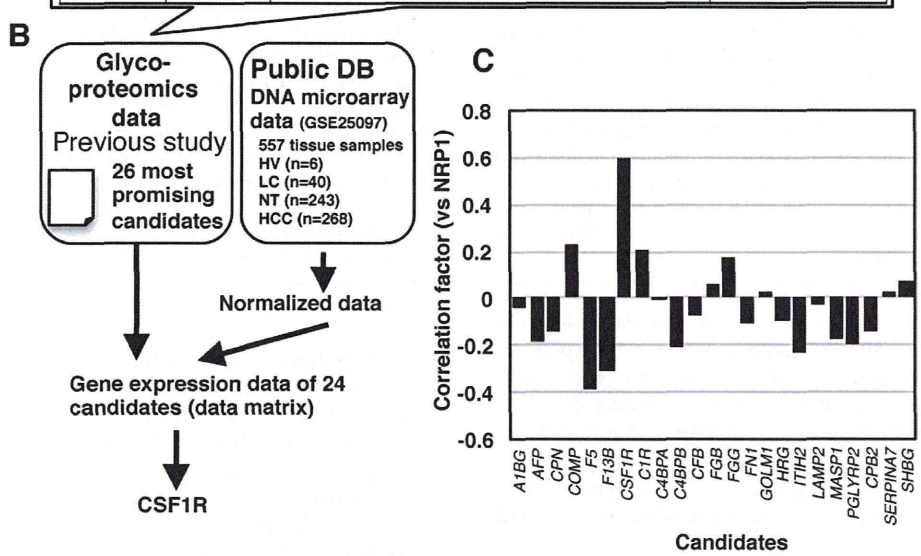

Figure 2. Selection of target molecule for the development of novel glycobiomarker candidate. (A) Twenty-six promising serum glycobiomarker candidates for liver disease. (B) Flow diagram outlining the strategy for candidate selection. (C) Correlation analysis of the expression of 23 candidate genes as compared to that of NRP1.

relationship between the levels of these markers and liver disease progression, we measured their levels in the serum of 141 patients whose fibrosis stages were diagnosed by biopsy (Table 2). Both the total CSF1R level and WFA$^+$-CSF1R level increased along with the progression of the liver disease (fibrosis stages F1–F3) (Figure 4C and D). The difference between F3 (n = 20) and F4 without HCC (n = 46) groups was higher for WFA$^+$-CSF1R ($P = 2.4 \times 10^{-6}$) than that for total CSF1R ($P = 5.5 \times 10^{-3}$). The difference between chronic hepatitis (F1–F3) (n = 74) and LC (F4) (n = 67) groups was higher for WFA$^+$-CSF1R ($P = 1.3 \times 10^{-17}$) than that for total CSF1R ($P = 5.7 \times 10^{-7}$). These results suggests that the WFA$^+$-CSF1R level is a more specific indicator of LC than the total CSF1R level. There was little difference between the levels of total CSF1R and WFA$^+$-CSF1R in F4 with HCC and F4 without HCC. Thus, the onset of HCC had no effect on the levels of total CSF1R and WFA$^+$-CSF1R.

Expression Profiles of WFA Epitope and CSF1R in Liver Tissues

To determine the expression profiles of the WFA epitope and CSF1R, we performed immunohistochemical analysis using liver tissue samples from LC patients. Although CSF1R is known to be expressed in and around the hepatic parenchymal cells in HCC,[20] we found that the expression of CSF1R is strongly and predominantly localized at the regenerating nodule, especially at the marginal region. In this region, CSF1R was expressed by hepatic parenchymal cells and macrophages in the sinusoid (Figure 5A). WFA epitopes were also expressed by hepatic parenchymal cells in the

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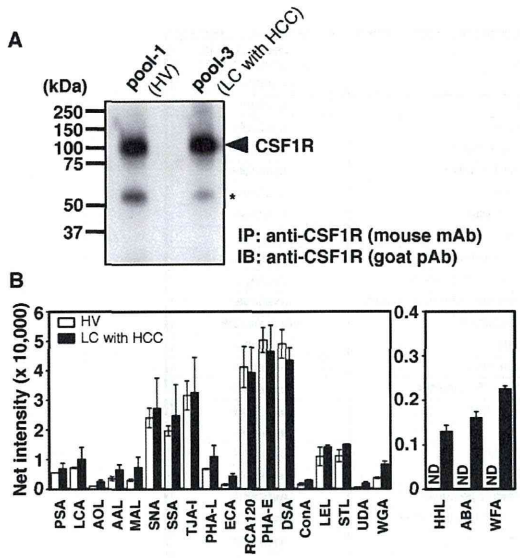

Figure 3. Differential glycan profiling of serum CSF1R. Pooled sera from HV and LC with HCC patients were subjected to differential glycan profiling. (A) Immunoprecipitated serum CSF1R was detected by Western blotting. IP, antibody for immunoprecipitation (mouse monoclonal anti-CSF1R antibody, mouse mAb); IB, antibody for immunoblotting (goat polyclonal anti-CSF1R antibody, goat pAb). Asterisk, nonspecific band of IgG. (B) Immunoprecipitated CSF1R samples from pooled sera (pool-1, pool-2, pool-3, and pool-4) were subjected independently to ALP assay as described under Materials and Methods section. The open bars represent the mean intensities of the CSF1R derived from pool-1 and pool-2 (HV), and shaded bars represent the mean intensities of CSF1R derived from pool-3 and pool-4 (LC with HCC). The standard deviations are indicated as whiskers. ND, not detected.

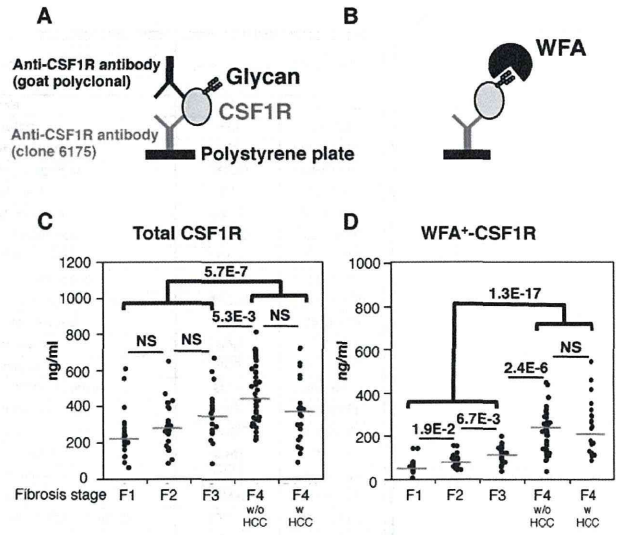

Figure 4. Verification of total CSF1R and WFA$^+$-CSF1R. (A) An image of the sandwich ELISA system used for the verification study of total CSF1R. Serum CSF1R binds to the capture antibody, and the detection antibody recognizes the peptide epitope(s) of CSF1R. (B) A cartoon of the sandwich ELISA system used for the verification study of WFA$^+$-CSF1R. Serum CSF1R binds to the capture antibody, and WFA recognizes the glycan epitope(s). Levels of total CSF1R were determined by Ab-Ab ELISA (C), and levels of WFA$^+$-CSF1R were determined by Ab-WFA ELISA (D). Levels of total CSF1R and WFA$^+$-CSF1R in hepatitis C (liver fibrosis stages F1–F3), LC without HCC (F4 without HCC), and LC with HCC (F4 with HCC) patients are shown on a scatter plot. The medians for each group are shown as bars in each graph. Significant difference between each group was determined by Student's t test for total CSF1R and by the Mann–Whitney U test for WFA$^+$-CSF1R. The P values (<0.05) are indicated for each plot. NS represents not significant.

marginal region of the regenerating nodule and macrophages in the sinusoid (Figure 5B). In addition, some cells expressing WFA epitopes were observed in the fibrous tissue (Figure 5B). Thus, CSF1R and WFA epitopes were coexpressed in the marginal region of the regenerating nodule and macrophages (Figure 5C). These results suggested the possibility that the serum WFA$^+$-CSF1R is derived from the hepatic parenchymal cells in the marginal region of the regenerating nodules and macrophages.

Comparison Analysis between CSF1R and M2BP

As described above, WFA$^+$-CSF1R was discovered in accordance with the strategy proposed by Narimatsu et al.[15] through its proof-of-concept experiment. Regarding the other, we have previously reported WFA$^+$-M2BP, which was discovered using another strategy of glycobiomarker development,[23] as a promising fibrosis marker. Therefore, we preliminarily evaluated the feasibility of these marker candidates using the sample set available in this study. To compare the levels of WFA$^+$-CSF1R and WFA$^+$-M2BP in serum, a correlation analysis was performed using the results on all samples described in Table 2. These results were plotted as shown in Figure 6, and regression analysis of the 2D plot indicated that the correlation coefficient (R^2) was 0.59. The outliers on the regression line included LC patients with poor prognosis (data not shown). Although these results showed a correlation between WFA$^+$-M2BP and WFA$^+$-CSF1R, there were some cases that deviated from the majority or remained independent.

As there were similarities and a few differences between the levels of WFA$^+$-CSF1R and WFA$^+$-M2BP, we compared localization of CSF1R and M2BP in the regenerating nodule. CSF1R was found both in the hepatic parenchymal cells and macrophages of this region (Figure 7A). In the hepatic parenchymal cells, diffused localization of CSF1R was observed. Although M2BP was also found both in the hepatic parenchymal cells and macrophages (Figure 7B), only granular localization of M2BP was observed in the hepatic parenchymal cells. Thus, CSF1R and M2BP were coexpressed in macrophages and parenchymal cells (Figure 7C). These observations suggest that even though CSF1R and M2BP were derived from the same cells, their localized forms or secretory mechanisms might be different.

DISCUSSION

In general, development of a disease-specific serological protein biomarker is very difficult,[15] as very few proteins would be secreted exclusively from a single lesion. Even if the level of a serum protein increased under a diseased condition, this increment may not be exclusively due to the lesion.[24] Disease-specific alterations in the glycan structures (disease-related glycoform) of specific proteins are recently receiving attention as the target for biomarker discovery. Evidence obtained from several previous studies suggested that disease-related glycoforms from specific lesions do have advantages in diagnostic specificity.[7−9,23,25,26] In the present study, we showed that the

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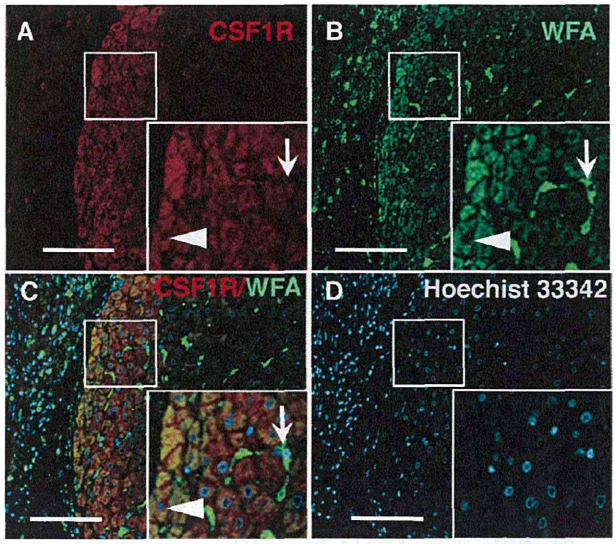

Figure 5. Histochemical analysis of CSF1R expression and WFA-epitope expression in LC tissue. (A) CSF1R was stained with anti-CSF1R and visualized by Alexa Fluor 546 (red). (B) WFA-epitope was stained with biotinylated WFA and visualized Alexa Fluor 488 (green). (C) The merged image shows colocalization of CSF1R and WFA epitope (yellow). (D) The nucleus was stained with Hoechst 33342 (blue). Scale bar, 100 μm. Arrow-head, parenchymal hepatic cell. Arrow, macrophage.

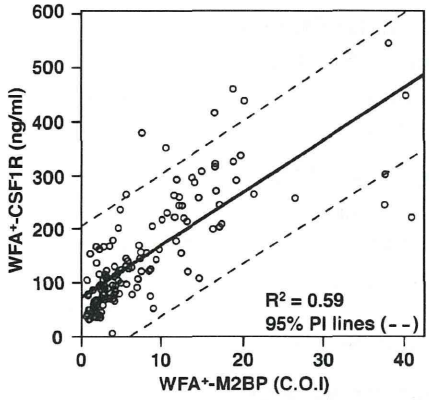

Figure 6. Correlation between WFA$^+$-CSF1R and WFA$^+$-M2BP. Correlation analysis was performed using a two-dimensional scatter plot. The serum WFA$^+$-CSF1R level (y-axis) is compared to the cut off index (C.O.I.) of WFA$^+$-M2BP (x-axis). Data obtained from each patient are indicated using an open circle. The linear correlation between the WFA$^+$-CSF1R and WFA$^+$-CSF1R values is represented by the regression curve, $y = 71.4 + 9.7x$ (solid line). The upper and lower 95% prediction intervals (PI lines) are represented by broken line.

liver disease-related increase of serum WFA$^+$-CSF1R, one of the glycoproteins carrying a disease-related glycoform, was more specific than that of the total CSF1R. These results support the idea that a specific glycoprotein carrying a disease-related glycoform could serve as a good target for developing novel biomarkers.

LC has been described as the terminal phase of hepatitis.[27] Progression of LC is illustrated by accompanying symptoms of liver failure such as ascites, esophageal varix due to portal hypertension, and the Child-Pugh score, which is calculated based on these symptoms and has been widely used as a

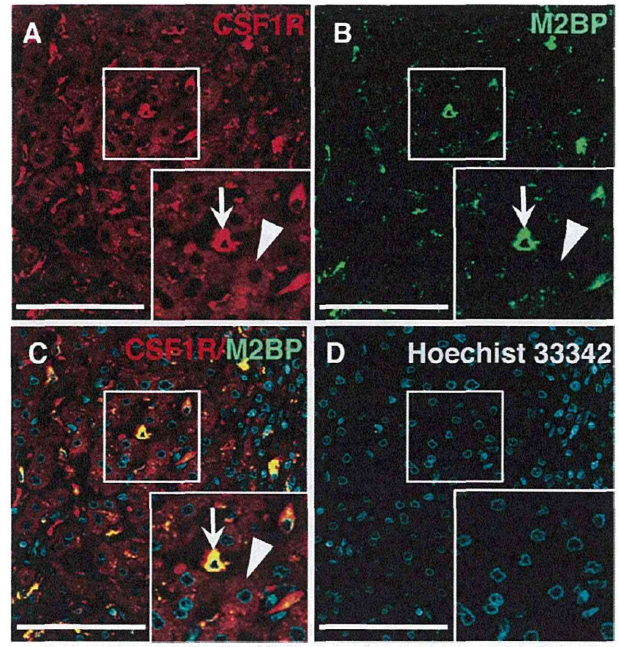

Figure 7. Immunohistochemical analyses of CSF1R and M2BP. (A) CSF1R was stained with anti-CSF1R and visualized by Alexa Fluor 546 (red). (B) M2BP was stained with biotinylated WFA and visualized by Alexa Fluor 488 (green). (C) The merged image shows colocalization of CSF1R and M2BP (yellow). (D) The nucleus was stained with Hoechst 33342 (blue). Scale bar, 100 μm. Arrow-head, parenchymal hepatic cell. Arrow, macrophage.

reference for the prognosis of LC patients.[28,29] However, more markers, which could possibly indicate the progression of LC and prognosis of LC patients, would be required in clinical practice.[30] In a preliminary retrospective analysis using a small number of samples (n = 42), when the LC patients were grouped on the basis of the serum WFA$^+$-CSF1R level, fewer patients survived 5 years of follow-up in the high WFA$^+$-CSF1R group (data not shown). The result suggested that the serum WFA$^+$-CSF1R level might be applicable for prognosis prediction of liver cirrhosis. However, as the results of clinical studies often change with the number of subjects, detailed clinical analyses with controlled and larger samples are necessary as the further studies.

The results obtained from our study, designed for the verification of the possible biomarker candidate using a limited number of samples, suggested that high WFA$^+$-CSF1R level is associated with progression level of LC. One of the possible mechanisms for enhanced production of CSF1R is activation of macrophages. Kupffer cells, the sinusoidal cells in the liver, are known to be involved in LC development.[31,32] When macrophages are activated, the scavenger receptor CD163 is cleaved by a PKC-mediated proteolytic mechanism and is secreted into the circulating blood.[33,34] The high level of serum CD163 is associated with poor prognosis of LC.[35] Likewise, CSF1R is also known to be secreted from the activated macrophages by a PKC-mediated mechanism.[22] The expression pattern of CSF1R in the liver tissue of LC patients (Figure 5) suggested that the secretion of CSF1R into the serum is caused by the activation of macrophages. On the other hand, regeneration of hepatocytes is also attributable to the CSF1R secretion, because CSF1R expression was observed in the

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parenchymal cells of the regenerating nodule. Since CSF1R is a signaling factor that promotes cell proliferation,[36] there ought to be a relationship between the cell proliferation at the regenerating nodule and the CSF1R expression. These mechanisms occurring in the cirrhotic liver are believed to be the cause for the secretion of CSF1R in the serum.

In contrast to the present study, our recent study suggested that WFA$^+$-M2BP is a highly accurate and reliable fibrosis marker.[23] This study was performed using a methodology different from the one we used in the present study,[16] wherein we combined glycoproteomics with the IGOT-LC–MS technology. M2BP was selected because of its previously known characteristics: the level of M2BP in the serum is generally high and increases along with the progression of liver fibrosis[37] and forms highly glycosylated multimers.[38] That previous report on M2BP demonstrated the utility of the highly sensitive lectin microarray system for developing functional glycobiomarkers using an automated measuring system for WFA$^+$-M2BP. In contrast, in the present study, we have developed WFA$^+$-CSF1R by systematic use of -omics technologies, hence revealing the practical value of our systematic strategy.

Comparative analysis of the levels of WFA$^+$-M2BP and WFA$^+$-CSF1R (Figure 6) suggested that both of them are useful for detecting progression of the liver disease. Since immunohistochemical staining showed colocalization of CSF1R and M2BP in most cells of the regenerating nodules, especially in macrophages (Figure 7), both CSF1R and M2BP may reflect, at least partly, similar disease status of the liver. Although we found a correlation between WFA$^+$-CSF1R and WFA$^+$-M2BP (Figure 6), the presence of a few outlier cases suggest that they may have distinctive characteristics that could differentiate between them. Functional differences between these two proteins may contribute to their serum levels. Since M2BP is a secretory protein, its expression level should be readily reflected in the serum from the early stage of liver fibrosis. Thus, WFA$^+$-M2BP has been reported as a good serum marker reflecting liver fibrosis. CSF1R, on the other hand, is a membrane protein that functions as a signaling factor, and the mechanism for its secretion is already known. It is considered that CSF1R derived from parenchymal hepatic cells is released by proteolytic events through the microenvironmental aggravation of liver tissue of LC. Thus, WFA$^+$-CSF1R could serve as a possibly direct index for the pathological changes attributable to disease malignancy. It would be essential to conduct further comparative analyses between WFA$^+$-M2BP and WFA$^+$-CSF1R to shed further light on how these two molecules could be better utilized for monitoring the liver disease. Incidentally, we identified WFA as the common factor for both molecules. Similar to the previous study,[23] our strategy described in this study led us to WFA as the "low-noise" lectin, as opposed to the "high-noise" fucose-binding lectin, for use in the direct measurement assay. These results clearly indicate that WFA could be used in the identification phase of glycobiomarker development. Instead of the extensively used methods of identification for fucosylated glycoproteins ("Fuc-omics"), adopting global proteomic methods for identification of glycoproteins in the WFA-bound fraction of cellular extracts, culture supernatants, or sera ("WFA-omics") could be a clever and innovative idea.

Kaji et al. identified the glycobiomarker candidates for liver diseases based on comparative glyco-profiling of serum samples from HV and patients with HCC accompanied by LC. Because the samples at the end stage of disease were used for the analysis, it was not clear if the candidates have specificity against each stage of the liver disease progression in the previous report.[16] In this study, we propose that the WFA$^+$-CSF1R is a possible biomarker candidate for evaluation of LC. In the future, via further clinical study, it is expected that HCV-infected patients who are in a progressive state of LC could be identified by using the WFA$^+$-CSF1R test. A combination of Peg-IFN and ribavirin is generally recommended as anti-HCV therapy for chronic hepatitis patients.[39] While this therapy had not been recommended for LC patients, some reports suggest that favorable outcome was observed in some cases of LC.[40] Considering the cost benefit and adverse effect of the anti-HCV therapy, evaluation of the therapeutic effect based on the liver function before and during the therapy is very useful, where the serum WFA$^+$-CSF1R level could serve as indices. We are planning to conduct further studies for evaluating therapeutic eligibility of LC patients and implementation of appropriate therapeutic strategies by monitoring their WFA$^+$-CSF1R level.

The serum WFA$^+$-CSF1R test described in this study was developed rationally through comprehensive identification of candidate molecules[15] by combinatorial use of glycoproteome, efficient selection of promising candidate molecules, and efficient glycome analysis. Development of a possible glycobiomarker candidate through such systematic approach strongly suggests that our strategy is indeed effective. Thus, we believe that this combined approach could be applied to search for other valuable serum biomarker for unmet medical needs.

■ 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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