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

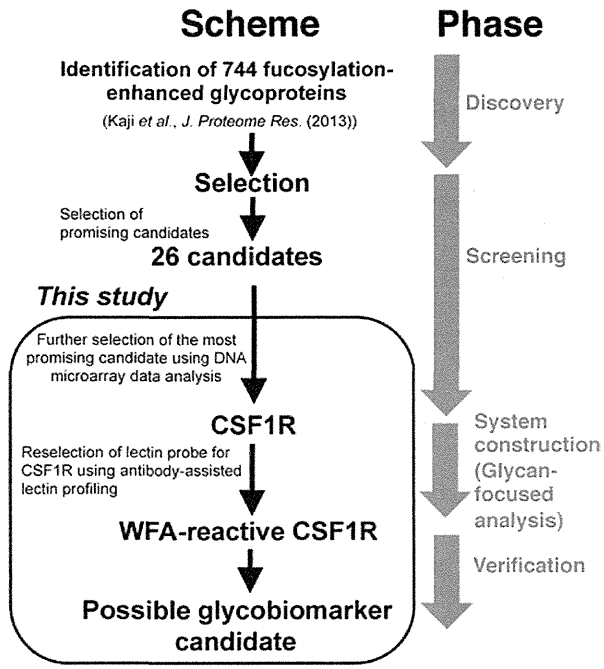

Figure 1. Schematic flow diagram outlining the strategy for glycobiomarker development. We previously identified 744 glycobiomarker candidates, and from these putative candidates we selected 26 serum glycoproteins as the promising candidates (discovery phase[23]). In this study, we screened these 26 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).

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.

Selection for 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

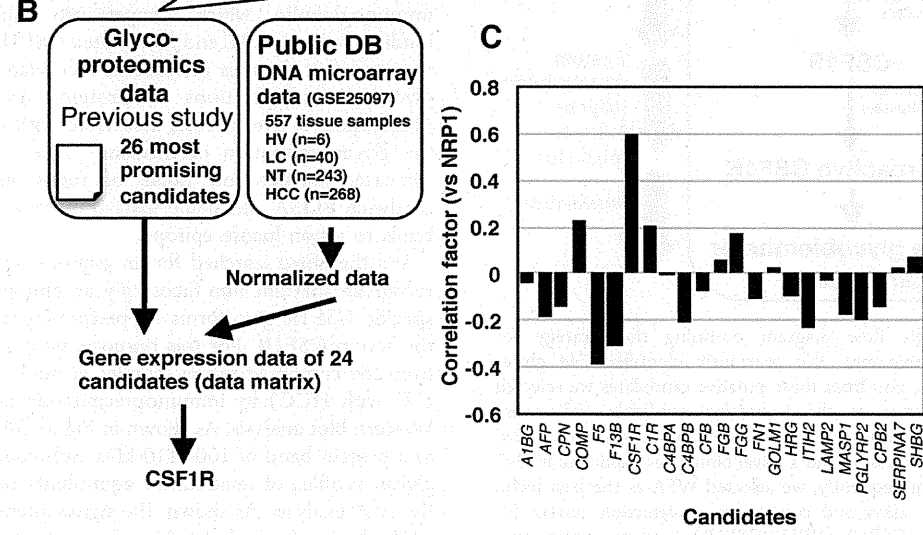

Figure 2. Selection of target molecule for the development of novel glycobiomarker candidate. (A) Twenty-six promising serum glycobiomarker candidates for liver disease. (B) Flow diagram outlining the strategy for candidate selection. (C) Correlation analysis of the expression of 23 candidate genes as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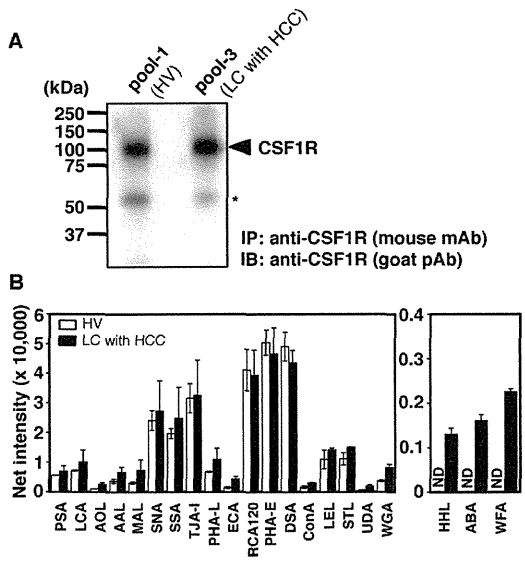

Figure 3. Differential glycan profiling of serum CSF1R. Pooled sera from HV and LC with HCC patients were subjected to differential glycan profiling. (A) Immunoprecipitated serum CSF1R was detected by Western blotting. IP, antibody for immunoprecipitation (mouse monoclonal anti-CSF1R antibody, mouse 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.

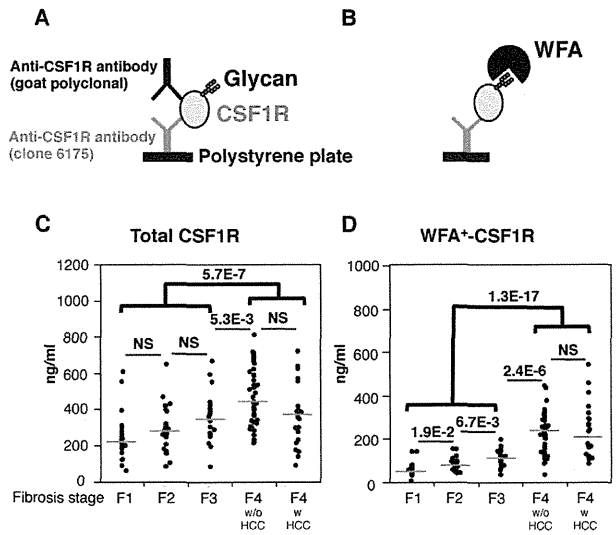

Figure 4. Verification of total CSF1R and WFA$^+$-CSF1R. (A) An image of the sandwich ELISA system used for the verification study of total CSF1R. Serum CSF1R binds to the capture antibody, and the detection antibody recognizes the peptide epitope(s) of CSF1R. (B) A cartoon of the sandwich ELISA system 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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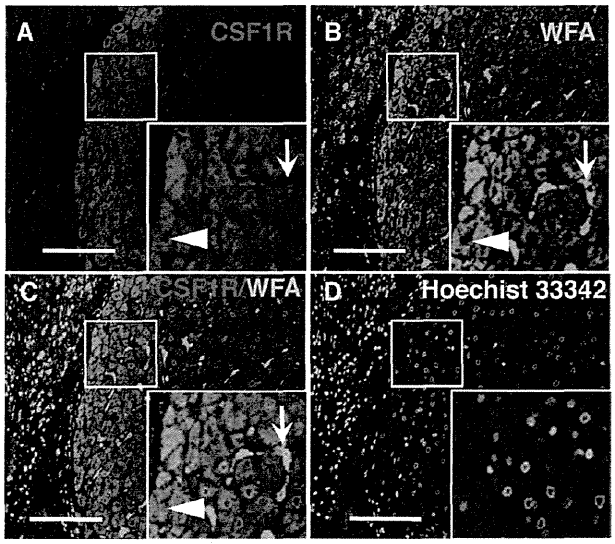

Figure 5. Histochemical analysis of CSF1R expression and WFA-epitope expression in LC tissue. (A) CSF1R was stained with anti-CSF1R and visualized by Alexa Fluor 546 (red). (B) WFA-epitope was stained with biotinylated WFA and visualized Alexa Fluor 488 (green). (C) The merged image shows colocalization 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.

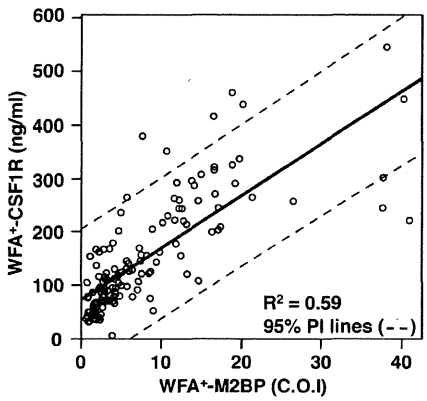

Figure 6. Correlation between WFA$^+$-CSF1R and WFA$^+$-M2BP. Correlation analysis was performed using a two-dimensional scatter plot. The serum WFA$^+$-CSF1R level (y-axis) is compared to the cut off index (C.O.I.) of WFA$^+$-M2BP (x-axis). Data obtained from each patient are indicated using an open 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

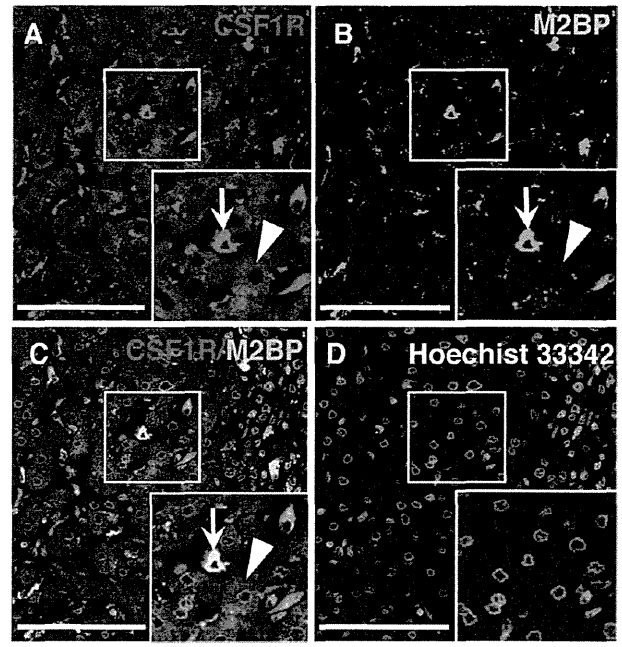

Figure 7. Immunohistochemical analyses of CSF1R and M2BP. (A) CSF1R was stained with anti-CSF1R and visualized by Alexa Fluor 546 (red). (B) M2BP was stained with biotinylated WFA and visualized by Alexa Fluor 488 (green). (C) The merged image shows colocalization of CSF1R and M2BP (yellow). (D) The 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

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Notes

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Alcohol and Smoking Affect Risk of Uncomplicated Colonic Diverticulosis in Japan

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Abstract

Colonic diverticula are located predominantly on the right side in Asia and on the left side in Europe and the United States. Factors associated with uncomplicated colonic diverticulosis and its distribution pattern have been unknown. Our aims are to investigate the prevalence and risk factors for uncomplicated colonic diverticulosis. We conducted a prospective cross-sectional study in adults who underwent colonoscopy. Alcohol, alcohol related flushing, smoking, medications, and comorbidities were assessed by interview on the colonoscopy day. Alcohol consumption was categorized as nondrinker, light (1–180 g/week), moderate (181–360 g/week), and heavy (≥361 g/week). Smoking index was defined as the number of cigarettes per day multiplied by the number of smoking years and categorized as nonsmoker, <400, 400–799, and ≥800. A total of 2,164 consecutive patients were enrolled. Overall, 542 patients (25.1%) had uncomplicated colonic diverticulosis located on the right side (50%), bilaterally (29%), and on the left side (21%). Univariate analysis revealed age, male, smoking index, alcohol consumption, aspirin use, anticoagulants use, corticosteroid use, hypertension, and atherosclerotic disease as factors significantly associated with diverticulosis. Alcohol related flushing was not associated with the disease. Multivariate analysis showed increasing age ($P<0.01$), increasing alcohol consumption ($P<0.01$) and smoking ($P<0.01$), and atherosclerotic disease ($P<0.01$) as significantly associated factors. Alcohol and smoking were associated with right-sided and bilateral diverticula. In conclusion, one in four Japanese adults have colonic diverticulosis (50% right-sided). Age, alcohol consumption, and smoking were found to be significant risk factors for uncomplicated colonic diverticulosis, particularly right-sided and bilateral.

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Introduction

Colonic diverticulosis shows geographic variation in both prevalence and pattern. Diverticulosis is rare in Africa and Asia, but common in the United States, Europe, and Australia [1–4]. The anatomic distribution pattern of diverticulosis is predominantly left-sided in the West and right-sided in Asia [1,4,5].

In Japan, the prevalence of colonic diverticulosis was 2.1% in the 1960s, but increased to 28% by 1997 [6–9]. Recent studies have shown that the current prevalence in Korea is 12% [10]. The increased prevalence of diverticulosis in Asian countries suggests that environmental and lifestyle factors play an important role in its pathogenesis [1,4,10].

Because asymptomatic colonic diverticulosis has potential to cause serious complications, such as diverticular bleeding or diverticulitis, it is crucial to understand the true prevalence and risk factors of the disease to prevent associated morbidity and mortality. However, the exact risk factors for uncomplicated colonic diverticulosis other than age remain unknown. Although constipation and low-fiber diet have been widely accepted as etiological factors for uncomplicated diverticulosis [1,4,11,12], a recent study showed that a high bowel movement frequency and a high-fiber diet were associated with a higher prevalence of diverticulosis [13]. Furthermore, a prospective cross-sectional study in Korea and the United States revealed alcohol consumption as a new risk factor for uncomplicated diverticulosis [10,14]. Therefore, further exploration of diverticulosis risk factors is needed.

The widespread use of colonoscopic examination has enabled increased detection of colonic diverticulosis [10], but few studies have reported on the specific factors associated with diverticulosis, especially in Asia [6,10]. To investigate the prevalence and to identify possible associated factors or risk factors of diverticulosis, we analyzed comprehensive data obtained from a prospective colonoscopy-based study that collated detailed information on smoking, alcohol consumption, comorbidities, and medications.

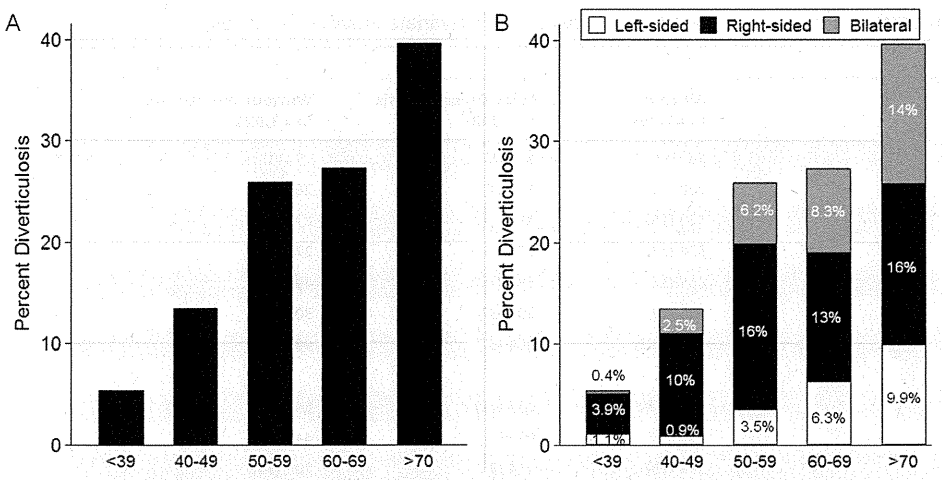

Figure 1. Prevalence of diverticulosis (A) and anatomic distribution (B) by age category (n = 2,164). Colonic diverticulosis increased with age (A). The prevalence of right-sided diverticula was high at for younger age, while left-sided and bilateral types increased with age (B).
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Materials and Methods

Study design, setting, and participants

We conducted a prospective cross-sectional single-center study in adults who underwent diagnostic colonoscopy between September 2009 and July 2012 at the endoscopy unit of the National Center for Global Health and Medicine (NCGM). The NCGM is an emergency hospital with 900 beds located in metropolitan Tokyo, Japan. The institutional review board at NCGM approved this study (No. 750) and all clinical procedures conformed to Japanese and International ethical guidelines (Declaration of Helsinki). All patients gave informed written consent prior to enrolment. No ethical problems exist with regard to the publication of this manuscript. We used anonymized data from patient medical records.

Inclusion criteria were as follows: (1) >18 years old; (2) Japanese nationality; (3) independence in activities of daily living; (4) able to understand written documents; (5) able to write; (6) asymptomatic patients who needed examination for colorectal cancer due to increasing tumor marker and/or fecal occult blood test results and/or abnormal findings on abdominal ultrasonography, computed tomography (CT), positron emission tomography-computed tomography (PET-CT), magnetic resonance imaging (MRI); or patients who wanted screening for colorectal cancer. Exclusion criteria were as follows: (1) patients who did not provide informed consent; (2) patients in whom total colonoscopy could not be performed; (3) and history of colon resection; (4) acute colonic diverticular bleeding or diverticulitis; (5) severe, continuous, or intermittent gastrointestinal (GI) symptoms such as frequent watery diarrhea and hematochezia within one week of onset to determine appropriate medical treatment. All inclusion and exclusion criteria were fulfilled before patients were enrolled.

Variables, Data sources, and Measurement

After informed consent was obtained, a detailed questionnaire was completed at the endoscopy unit on the same day as pre-colonoscopy. Patients were asked about their 1) lifestyle habits, 2) medications, and 3) comorbidities in a face-to-face interview with medical staff. For medication history, prescriptions and medical records were reviewed in addition to information provided by the patients to avoid omissions.

Patients were asked the following four questions regarding alcohol consumption: "*Do you drink alcohol?*", "*What types of alcohol do you usually drink; for example, beer, shochu, sake, wine, gin, vodka, whiskey, tequila, or brandy?*", "*How many days per week do you drink alcohol?*", and "*How many glasses of about 180 ml of alcohol do you usually drink per day?*" Then, alcohol consumption was calculated and subjects were categorized as nondrinker, light drinker (1–180 g/week), moderate drinker (181–360 g/week), and heavy drinker (≥361 g/week). Duplicate data were allowed.

The flushing questions consisted of the following two items: "*Do you have a tendency to develop facial flushing immediately after drinking a glass of about 180 ml of beer?*" and "*Did you have a tendency to develop facial flushing immediately after drinking a glass of beer in the first one or two years after you started drinking alcohol?*" For both questions, the choice of answers was "*yes*", "*no*", and "*unknown*". If a subject answered yes to either question, they were considered to be deficient in acetaldehyde dehydrogenase 2 (ALDH2) [15].

The smoking index was evaluated among ever and daily smokers and was defined as the number of cigarettes per day multiplied by the number of smoking years. Then, smoking index was categorized as nonsmoker, <400, 400–799, and ≥800.

Patients were asked about regular use of aspirin, anticoagulants, and oral corticosteroids (prednisolone, methylprednisolone, betamethasone, dexamethasone, or hydrocortisone). The survey form included photographs of these oral drugs, which are approved in Japan. Regular use of medication was defined as oral administration starting at least 1 year before the interview.

Evaluated comorbidities were hypertension, atherosclerotic vascular disease including diabetes mellitus and dyslipidemia, coronary heart disease, and chronic renal failure. Diabetes mellitus, dyslipidemia, and hypertension were considered present in patients taking specific drugs. Chronic renal failure was considered present in patients on hemodialysis or peritoneal dialysis, or with serum creatinine levels ≥2.0 mg/dl.

An electronic high-resolution video endoscope (model CFH260; Olympus Optical, Tokyo, Japan) was used for diagnosis of colonic diverticula. Intestinal lavage for endoscopic examination was performed using 2 L of solution containing polyethylene glycol. If diverticula were observed within the colon, their location type was recorded in the electronic endoscopic database. Distribution type was defined as follows: right-sided, involving the splenic flexure,

Table 1. Characteristics in patients with or without colonic diverticulosis on univariate analysis (n = 2,164).

Variables	All cases (n = 2,164)	With Diverticulosis (n = 542)	Without diverticulosis (n = 1,622)	P
Mean Age (SD), years	58 (14)	56 (15)	65 (11)	<0.01
<39	280 (13)	15 (2.8)	265 (16)	
40–49	320 (15)	43 (7.9)	277 (17)	
50–59	374 (17)	97 (18)	277 (17)	
60–69	685 (32)	187 (35)	498 (31)	
>70	505 (23)	200 (37)	305 (19)	<0.01
Sex (Male)	1,356 (63)	364 (67)	992 (61)	0.01
Smoking index*				
Nonsmoker	1,056 (49)	214 (39)	842 (52)	
<400	533 (25)	93 (17)	440 (27)	
400–799	319 (15)	114 (21)	205 (13)	
>800	256 (12)	121 (22)	135 (8.3)	<0.01
Alcohol				
Non-drinker	856 (40)	142 (26)	714 (44)	
Drinker	1,308 (60)	400 (74)	908 (56)	<0.01
Alcohol consumption				
Non-drinker	856 (40)	142 (26)	714 (44)	
Light drinker (1–180 g/week)	983 (45)	270 (50)	713 (44)	
Moderate drinker (181–360 g/week)	207 (9.6)	69 (13)	138 (8.5)	
Heavy drinker (≥361 g/week)	118 (5.5)	61 (11)	57 (3.5)	<0.01
Alcohol Flusher				
Non-flusher or unknown	1708 (79)	434 (80)	1274 (79)	
Flusher	456 (21)	108 (20)	348 (21)	0.45
Medication				
Regular aspirin use	206 (9.5)	80 (15)	126 (7.8)	<0.01
Regular anticoagulants use	103 (4.8)	40 (7.4)	63 (3.9)	<0.01
Regular corticosteroid use	170 (7.9)	26 (4.8)	144 (8.9)	<0.01
Comorbidity				
Hypertension	745 (34)	264 (49)	481 (30)	<0.01
Atherosclerotic vascular disease	644 (30)	223 (41)	421 (26)	<0.01

Categorical variables are reported as n (%).
*The smoking index was evaluated among ever and daily smokers and was defined as the number of cigarettes per day multiplied by the number of smoking years.
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transverse or proximal colon; left-sided, involving the descending or distal colon; or bilateral, involving the entire colon.

Statistical analysis

Patients with colonic diverticula were defined as subjects, and those without colonic diverticula were defined as controls, and the relationships between colonic diverticula and clinical factors were examined. To determine risk factors for colonic diverticula, we estimated the odds ratio (OR) and 95% confidence interval (CI). Pearson's Chi-squared test was used to determine the univariate association between each variable and the presence of diverticulosis. In multivariate analysis, we used a multiple logistic regression model. The Cochran–Armitage test was used to identify the trend between each variable and the presence of diverticulosis. A value of $P<0.05$ was considered significant. All statistical analysis was performed using Stata version 10 software (StataCorp, College Station, Texas, USA).

Results

Participants and prevalence

During the study period, 2,319 patients participated in medical interviews. Of them, 91 could not undergo total colonoscopy and 64 had a history of colorectal resection. Ultimately, 2,164 consecutive patients comprising 542 patients with uncomplicated colonic diverticula were enrolled. The overall prevalence of colonic diverticulosis was 25.0%. Diverticula were located predominantly in the right-side of the colon in 50.0% (n = 271), bilaterally in 29.3% (n = 159), and in the left-side in 20.7% (n = 112) of cases. The prevalence of colonic diverticulosis increased with age (Figure 1A). The prevalence of right-sided diverticula was significantly higher in younger patients than in older patients (<39 vs 40–59 years, $p<0.01$; 40–59 vs 50–59 years, $p = 0.02$), and tended to increase significantly ($P<0.01$ for trend) with age. Similarly, that for left-sided and bilateral types also tended to increase significantly ($P<0.01$ for trend) with age (Figure 1B). There were 223 patients (10.3%) who underwent on

Table 2. Factors associated with colonic diverticulosis on multivariate analysis.

Variables	Odds ratio (95% CI)	P
Age	1.1 (1.0–1.1)	<0.01
Sex		
Female	1 (referent)	
Male	1.0 (0.74–1.2)	0.75
Smoking index*		
Nonsmoker	1 (referent)	
<400	0.90 (0.66–1.2)	0.47
400–799	1.7 (1.3–2.4)	<0.01
>800	1.8 (1.3–2.5)	<0.01
Alcohol consumption		
Non-drinker	1 (referent)	
Light drinker (1–180 g/week)	2.2 (1.7–2.8)	<0.01
Moderate drinker (181–360 g/week)	2.7 (1.8–4.0)	<0.01
Heavy drinker (≥361 g/week)	5.6 (3.6–8.8)	<0.01
Alcohol Flusher		
Non-flusher or unknown	1 (referent)	
Flusher	0.87 (0.66–1.1)	0.30
Medication		
Regular aspirin use		
No	1 (referent)	
Yes	1.1 (0.75–1.5)	0.73
Regular anticoagulants use		
No	1 (referent)	
Yes	1.4 (0.88–2.2)	0.16
Regular corticosteroid use		
No	1 (referent)	
Yes	0.69 (0.43–1.1)	0.11
Comorbidity		
Hypertension		
No	1 (referent)	
Yes	1.2 (0.95–1.5)	0.13
Atherosclerotic vascular disease		
No	1 (referent)	
Yes	1.4 (1.1–1.8)	<0.01

*The smoking index was evaluated among ever and daily smokers and was defined as the number of cigarettes per day multiplied by the number of smoking years.

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CT, PET-CT, or MRI. No significant differences in the prevalence of colonic diverticulosis were noted between the group with abnormal imaging findings and the group with normal imaging findings in 23.3% (n = 17/73) and 22.0% (n = 33/150) (P = 0.83) of cases, respectively.

Risk factors

Table 1 shows patient characteristics. On univariate analysis, age, male, smoking index, alcohol consumption, regular aspirin use, regular anticoagulants use, regular corticosteroid use, hypertension, and atherosclerotic disease were significantly associated with diverticulosis. On multivariate analysis, increasing age, increasing alcohol consumption and smoking, and atherosclerotic disease were significantly associated with diverticulosis (Table 2). Relative risk tended to increase with age, smoking index, and the amount of alcohol consumed (Table 2).

Distribution type and factors

Right-sided and bilateral diverticula increased significantly in line with alcohol consumption (P<0.01 for trend) (Figure 2A), while left-sided diverticula were not significantly (P = 0.60) associated with alcohol consumption (Figure 2A).

Distribution type of colonic diverticula increased significantly in line with smoking index (left-sided: P<0.01; right-sided: P<0.01; and bilateral diverticula: P<0.01; respectively for trend) (Figure 2B).

Discussion

In this colonoscopy-based study, we demonstrated that older age and alcohol consumption are strong risk factors for uncomplicated colonic diverticulosis, and the risk increases in line with the amount of alcohol consumed. Furthermore, patients with high pack-years of smoking, hypertension, and atherosclerotic vascular disease were found to be predisposed to colonic diverticulosis. The prevalence of right-sided and bilateral diverticula increased significantly as alcohol consumption and smoking increased.

Several studies have shown that the prevalence of colonic diverticulosis in Japan increased to 28% by 1997 [6–9]. Although no available data were available for the 2000s, we found that the prevalence for this period was 25%. To our knowledge, this is the first prospective study to identify the prevalence of colonic diverticulosis in Japan based on colonoscopic findings because most previous studies used barium enema [6–9]. Colonoscopy is used worldwide as a standard tool for the detecting colonic cancer and diverticulosis [10], but it can miss diverticula, especially those in the left-sided colon [16]. Thus, the prevalence of diverticula determined in this study is likely to be lower than the actual prevalence.

Age has been found to be an important risk factor for colonic diverticulosis. It has been suggested that patients with diverticular disease have greater rates of collagen cross-linking [17]. In addition, abnormal thickness of muscles of colonic wall, including collagen cross-linking, is promoted by abnormal colonic movement due to a lack of dietary fiber and results in increased intraluminal colonic pressure or fragility of the thickened muscles due to intraluminal pressure changes with age [3,4].

Consistent with past studies [4,5,8,9], the diverticula in our subjects first developed on the right-sided colon and extended to the left-sided and bilateral colon with aging. Right-sided diverticula in the Japanese has been considered to be of congenital origin [4], thus identification of factors associated with the right-sided type is important to understand the development of colonic diverticula. Why diverticulis is predominantly right-sided in Asian people and rarely so in other populations, who have the same risk factors as those identified in this study, is unclear. It is possible, however, that differences in the sensitivity of the colon to environmental factors are due to variations in characteristics such as the length and muscle thickness of the colon, body weight, and the structure of the neural and humoral systems [4].

In the present study, alcohol intake and amount were not only associated with the entire colonic diverticula but specifically with right-sided diverticula. Song et al. [10] revealed that alcohol drinkers were two times (OR: 2.2) more likely to develop diverticulosis than nondrinkers when assessed by multivariate analysis in a colonoscopy-based study. Sharaha et al. [14] recently

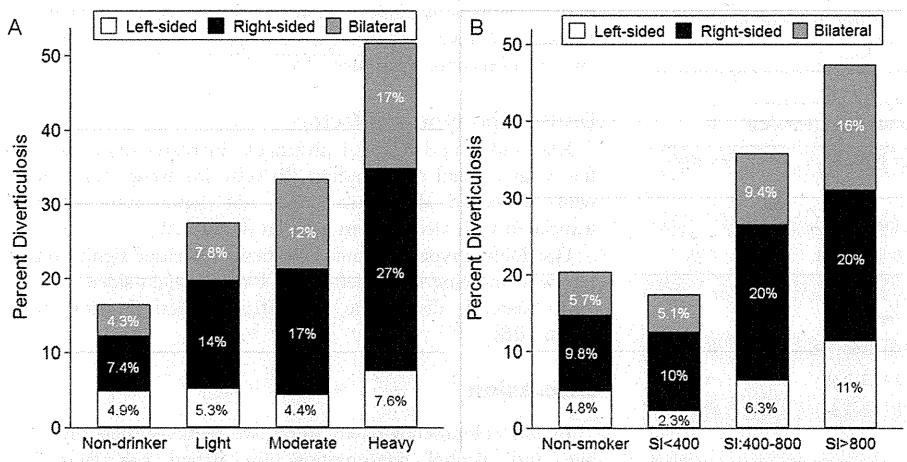

Figure 2. Prevalence of diverticulosis and anatomic distribution by alcohol consumption (A) and smoking index (B) (n = 2,164). Right-sided and bilateral diverticula increased significantly in line with amount of alcohol consumption (A). All distribution types of colonic diverticula increased significantly in line with smoking index (SI) (B).
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conducted a prospective colonoscopy-based study and found that the OR for diverticula was 1.96 with occasional alcohol use and 1.91 in a ≥1 drink per day group as a reference for non-drinkers. Indeed, alcohol intake is likely to have a deleterious effect for the development of diverticula, but no details on the amount of alcohol consumed were available for type of diverticula in their study. As we asked a detailed question with regard to type, times per week, and amount of alcohol, we were able to assess the precise consumption, which is a strength of this study. The biologic mechanisms linking alcohol to diverticulosis are unclear, but may involve colonic motility [18,19]. Berenson et al. [18] reported that intravenous administration of alcohol consistently decreases recto-sigmoid motor activity and correlates inversely with blood alcohol levels in humans. Wang et al. [19] demonstrated that alcohol inhibits colonic motility mainly through activation of NF-kB, subsequent upregulation of iNOS expression, and the increase of NO release in myenteric plexus in a rat model.

Smoking and the amount of pack-years was also found in this study to be another lifestyle risk factor for colonic diverticula and specifically the right-sided type. Only few data are available on the relationship between uncomplicated colonic diverticula and smoking [10,13]. Song et al. [10] found that smokers were 30% more likely to develop diverticulosis than nonsmokers after adjustment for important confounders, but this relationship was not statistically significant. Perry et al. [13] assessed smoking history defined as the total number of years smoked and found that patients with diverticulosis had longer tobacco use than those without. Possible mechanisms for the development of diverticulosis may include colonic microflora and colonic motility. Recently, colonic microflora has been shown to play an important role in the development and progression of diverticular disease [20]. Nicotine is known to inhibit the synthesis of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) [21], which may alter microflora. Furthermore, previous studies have shown that smoking increases chemical mediators such as vasoactive intestinal polypeptide (VIP) [22] and nitric oxide [23]. Milner et al. [24] revealed that the VIP content of the mucosa and whole wall was increased in diverticular disease. While, Tomita et al [25] reported that the colonic tissue of the diverticular-bearing segments is more strongly innervated by cholinergic nerves than normal segments of the colon. These findings suggest that chemical mediators affect colonic motility and intracolonic pressure, thereby possibly enhancing bulging of the colonic mucosa.

A limitation of this study is that several pathogenic factors reported to be associated with colonic diverticulosis were not included in the analysis; in particular, physical activity, familial and hereditary factors, obesity, and a detailed quantitative dietary history with regard to fiber and fat intake [3,4,12]. The absence of these factors could have confounded the relationships between alcohol and smoking. Although we demonstrated that the comorbidity of atherosclerotic vascular disease is associated with colonic diverticula on univariate analysis, this factor is not a true risk factor. We believe that patients with atherosclerotic vascular disease and colonic diverticula have common predisposing factors such as a low-fiber or high-fat diet and low physical activity.

In summary, our study shows that the overall prevalence of colonic diverticulosis was 25%, with 50% of cases on the right side. In addition to age, the amount of alcohol consumption and smoking were found to be identifiable risk factors for the development of uncomplicated colonic diverticulosis. These factors were also associated with right-sided and bilateral diverticula. Patients with atherosclerotic vascular disease are predisposed to colonic diverticula due to similar risk factors. Further study is needed to explore these associations as well as new risk factors from eastern and western countries.

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Author Contributions

Conceived and designed the experiments: NN TT JA MY MM NU TS. Performed the experiments: NN RN TA YK KS ST KW. Analyzed the data: TS. Contributed reagents/materials/analysis tools: RN TA YK KS ST KW NN. Wrote the paper: NN TI NU.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Ex vivo induction of IFN-λ3 by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients

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Abstract
Background Genetic variation around interleukin-28B (*IL28B*), encoding IFN-λ3, predict non-responders to pegylated interferon-α/ribavirin (Peg-IFN/RBV) therapy in chronic hepatitis C (CHC). However, it remains unclear the expression and the role of *IL28B* itself. The aim of this study is to develop easy and useful methods for the prediction of treatment outcomes.
Methods The mRNA and protein levels of IFN-λ3 induced by ex vivo stimulation of peripheral blood mononuclear cells (PBMC) or magnetically selected dendritic cells (DCs) with toll-like receptor agonists (TLR3; poly I:C, TLR7; R-837) were measured by the quantitative real-time polymerase chain reaction and our newly developed chemiluminescence enzyme immunoassays, respectively, and compared with the clinical data.
Results We found that BDCA-4$^+$ plasmacytoid and BDCA-3$^+$ myeloid DCs were the main producers of IFN-λs when stimulated with R-837 and poly I:C, respectively. Detectable levels of IFN-λs were inducible even in a small amount of PBMC, and IFN-λ3 was more robustly up-regulated by R-837 in PBMC of CHC patients with favorable genotype for the response to Peg-IFN/RBV (TT in *rs8099917*) than those with TG/GG. Importantly, the protein levels of IFN-λ3 induced by R-837 clearly differentiated the response to Peg-IFN/RBV treatment ($p = 1.0 \times 10^{-10}$), including cases that *IL28B* genotyping failed to predict the treatment response. The measurement of IFN-λ3 protein more accurately predicted treatment efficacies (95.7 %) than that of *IL28B* genotyping (65.2 %).
Conclusions Genetic variations around *IL28B* basically affect IFN-λ3 production, but different amounts of IFN-λ3 protein determines the outcomes of Peg-IFN/RBV treatment. This study, for the first time, presents compelling evidence that *IL28B* confer a functional phenotype.

Keywords Chronic hepatitis C · IL28B · IFN-λ3 · Peg-IFN/RBV

Abbreviations

ARFI	Acoustic radiation force impulses
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
IL28B	Interleukin-28B
Peg-IFN/RBV	Pegylated interferon-α/ribavirin
PBMC	Peripheral blood mononuclear cells
SNP	Single nucleotide polymorphisms
SVR	Sustained viral response
TLR	Toll-like receptor
TVR	Transient viral response
VR	Viral response

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Introduction

Recently, we and others independently identified single nucleotide polymorphisms (SNPs) on chromosome 19 associated with the interleukin-28B gene (*IL28B*), encoding IFN-λ3, that were strongly associated with the response to pegylated interferon-α/ribavirin (Peg-IFN/RBV) in chronic hepatitis C (CHC) patients, through a genome-wide association study (GWAS) [1–3]. According to our results, about 80 % of CHC patients with the TT genotype (*rs8099917*) showed viral virologic response (VR), including SVR (sustained virologic response) or TVR (transient virologic response), whereas only about 20 % of HCV patients with the TG/GG genotype showed VR [1]. Thus, by genotyping of *IL28B*, we can predict the efficacy of Peg-IFN/RBV before beginning treatment, avoiding unnecessary side effects and the high cost of Peg-IFN/RBV treatment. However, it is still unknown whether genetic variation of *IL28B* is a functional phenotype for Peg-IFN/RBV treatment. In addition, genotyping of *IL28B* alone failed to predict about 20 % of the response [1], which would be reasonable because final products of the genes are affected by DNA methylation or chromatin modifications as well as genetic variations [4].

Type III IFNs, consisting of IFN-λ1, λ2, and λ3 (also known as *IL29*, *IL28A* and *IL28B*, respectively), have recently been characterized [5, 6]. IFN-λs up-regulate IFN-stimulated genes (ISGs) via Janus kinase/signal transducer and activator of transcription (Jak/STAT) intracellular signaling, inhibiting hepatitis B virus (HBV) or hepatitis C virus (HCV) replication [7]. Antiviral responses evoked by toll-like receptor (TLR)3 or TLR9 agonists are attenuated in IL28RA$^{-/-}$ mice [8], indicating the central role of IFN-λs in antiviral protection. Clinically, early virologic response by Peg-IFN/RBV is associated with a high probability of SVR in HCV patients [9]. Genetic variations of *IL28B* influence spontaneous clearance of HCV [10], or on-treatment viral kinetics [11]. These results suggest a mechanistic link between innate immunity and genetic variations of *IL28B*.

To recognize viruses and trigger innate antiviral responses, mammals have 2 independent receptors, retinoic acid-induced gene-I (RIG-I)-like receptors (RLRs) and TLRs, distinct families of pattern recognition receptors that sense nucleic acids derived from viruses [12]. RIG-I is a double-stranded RNA-binding DExD/H box RNA helicase that is essential for initiating the intracellular response to RNA viral infection [13]. However, NS3/4A, the major serine protease expressed by HCV, disrupts the RIG-I pathway through proteolysis of essential signaling components of IFN regulatory factor 3 (IRF-3) activation [13, 14], reducing immune response. Alternatively, the TLR-families play an important role in innate immune responses in mammals [15]. Among them, TLR3 recognizes viral double-stranded RNA, whereas TLR7 recognize single-stranded RNA. Because some TLRs ligands induce IFN-λ in human macrophages [16], contributing to antiviral defense and HCV is a single-stranded RNA [8], we hypothesized that IFN-λ3 induced via the TLR pathway might contribute to early antiviral response against HCV, which could lead to accurate prediction of treatment efficacy. Therefore, we investigated IFN-λs production in peripheral blood mononuclear cells (PBMC) in healthy volunteers or CHC patients by ex vivo stimulation with TLR agonists, and analyzed whether this method could predict the responses to Peg-IFN/RBV treatment in clinical practice.

Patients, materials, and methods

Study population

Blood samples were obtained from 12 healthy volunteers and 100 consecutive Japanese outpatients with CHC (genotype 1b and high viral load) who visited our hospital between April 2011 and March 2012. The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of our institutes (NCGM-G-001023-01). Written informed consent was obtained from all volunteers and patients. All subjects were negative for HBV and human immunodeficiency virus, and did not have hepatocellular carcinoma. IFN treatment was not being given to any patient at the time blood samples were taken. The subjects were all evaluated for SNP near *IL28B* (*rs8099917*, *rs12979860*) using the InvaderPlus assay (Invader Chemistry, Madison, WI, USA) as previously reported [17].

Definition of treatment responses

Non-virologic response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks, and detectable viremia 24 weeks after initiation of treatment. VR was defined as achieving SVR or TVR. SVR was defined as undetectable HCV RNA in the serum 6 months after the end of treatment, whereas TVR was defined as reappearance of HCV RNA in the serum during or after completion of treatment.

Preparation of PBMC and selection of plasmacytoid or myeloid dendritic cells (DCs)

Whole blood anti-coagulated with EDTA was obtained from healthy volunteers and CHC patients. PBMC were isolated by Ficoll-Hypaque (Mediatech, Herndon, VA, USA) density

gradient centrifugation. BDCA-1, 3, 4^+DCs were negatively or positively selected by BDCA-1^+DC isolation kit, BDCA-3 MicroBead kit and BDCA-4/Neuropilin-1 MicroBead kit, respectively (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions.

Ex vivo induction of IFN-λ1, IFN-λ2, and IFN-λ3

After pre-treatment with or without 100 U/ml of IFN-α (Hayashibara Co. Ltd., Okayama, Japan) in 200 μl of Roswell Park Memorial Institutes (RPMI) medium supplemented with 10 % fetal bovine serum for 16 h, 100,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (TLR3 agonist; Imgenex, San Diego, CA, USA), or 5 μg/ml of imiquimod (R-837; TLR7 agonist, Imgenex) as previously reported [16]. For chemiluminescence enzyme immunoassays (CLEIA), 200,000 cells were subjected to the same stimulation protocol.

RNA isolation and cDNA synthesis

After stimulation with TRL-agonists for 4 h, the PBMC were lysed with ISOGEN-II (Nippon Gene, Tokyo, Japan). In some experiments, PBMC were harvested at each indicated time point. The lysate was supplemented with chloroform, incubated for 15 min on ice, and centrifuged at 22,000 g for 15 min. The aqueous layer was removed and precipitated with isopropanol. The RNA was pelleted by centrifugation, washed with ethanol, and dissolved in 20 μl of water. Reverse transcription was performed using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA).

Real-Time quantitative polymerase chain reaction (PCR)

Quantitative real-time PCR was performed to estimate IFN-λ1, IFN-λ2, and IFN-λ3 mRNA expression based on SYBR green fluorescence (Roche Diagnostics Japan), using TaqMan Universal PCR master mix (Roche Diagnostics Japan), according to the manufacturer's protocol. Relative gene expression was calculated as a fold induction. Data were analyzed using the $2\text{-}\Delta\Delta C(t)$ method with Sequence Detector version 1.7 software (Applied Biosystems, Carlsbad, CA, USA) and were normalized using human hypoxanthine phosphoribosyltransferase (HPRT). A standard curve was prepared by serial 10-fold dilutions of human cDNA. The curve was linear over 7 log units with a 0.998 correlation coefficient. Quantitative mRNA expression was determined by triplicate real-time PCR.

Chemiluminescence enzyme immunoassays

We recently developed a CLEIA system for IFN-λ3 that showed a wide detection range of 0.1–10,000 pg/ml with little or no cross-reactivity to IFN-λ1 or IFN-λ2 [18]. In addition, this CLEIA system can correctly detect IFN-λ3 from different *IL28B* genotypes.

Acoustic radiation force impulse (ARFI) elastography

For non-invasive evaluation of liver fibrosis, ARFI elastography was performed using a Siemens Acuson S2000™ ultrasound system (Mochida Siemens Medical System Co, Ltd, Tokyo, Japan) as previously reported [19]. We performed 5 measurements for each patient, and a median value was calculated. Liver stiffness was expressed as the shear wave velocity (m/s) and has been reported to be well correlated with histological liver fibrosis [19].

Statistical analyses

Continuous variables between groups were compared using the Mann–Whitney U test, and categorical data were compared using the Chi square test or Fisher's exact test. Correlations between continuous variables were searched using the Pearson correlation test. Values of $p < 0.05$ were considered significant.

Results

Genetic variation in *IL28B*

In CHC patients ($n = 100$), only 1 patient showed discrepancy between *rs8099917* and *rs12979860* with the same prediction for the treatment response by genotyping (TG in *rs8099917*, and TT in *rs12979860*). In addition, we recently reported that *rs8099917* has the greatest accuracy in determining the outcome of Peg-IFN/RBV treatment in Japanese patients [17]. Therefore, *rs8099917* is used in the following analyses. The major homologous (TT) in *rs8099917* is considered a predictive factor for a favorable response to Peg-IFN/RBV treatment, while having minor alleles (TG or GG) is considered predictive for non-responders. Seven of 12 healthy volunteers had the TT genotype of *IL28B* and 5 had TG genotype. In CHC patients, 59 patients had the TT genotype, 36 had TG, and 5 had GG in *rs8099917*.

BDCA-4^+ plasmacytoid DCs are the major producers of IFN-λs in response to R-837

Since Lauterbach et al. [20] found that human DCs expressing BDCA3 (CD141) in myeloid DC subsets were the primary producers of IFN-λs, we sought which cell types are the main producers of IFN-λs when stimulated with R-837. Because DCs from the different *IL28B*

genotype are supposed to produce different amounts of IFN-λs, we used DCs from healthy volunteers with TT genotype. After negative or positive magnetic selection of BDCA-1, 3, 4^+DCs using 100 ml of peripheral blood, each collection was stimulated with IFN-α, following poly I:C or R-837 as previously reported [16], and evaluated the mRNA of IFN-λs or the protein levels of IFN-λ3. We confirmed that BDCA-3^+DCs were the main producers of IFN-λs when stimulated with poly I:C as previously reported (Fig. 1a) [20]. Interestingly, when stimulated with R-837, positive selection of BDCA-4^+DCs (plasmacytoid DCs), not BDCA-3^+DCs, produced IFN-λs whereas depletion of BDCA-4^+DCs showed marked reduction of IFN-λs (Fig. 1b). Therefore, BDCA-4^+DCs were the main producers of IFN-λs when stimulated with R-837. Thus, different stimulation targeted different DC subsets to induce IFN-λs.

Induction of IFN-λs (IFN-λ1, IFN-λ2, and IFN-λ3) in PBMC from healthy volunteers

We confirmed that the main producers of IFN-λs were DCs. However, analyses of IFN-λs using DC subsets need a lot of blood, which cannot apply to patients, because

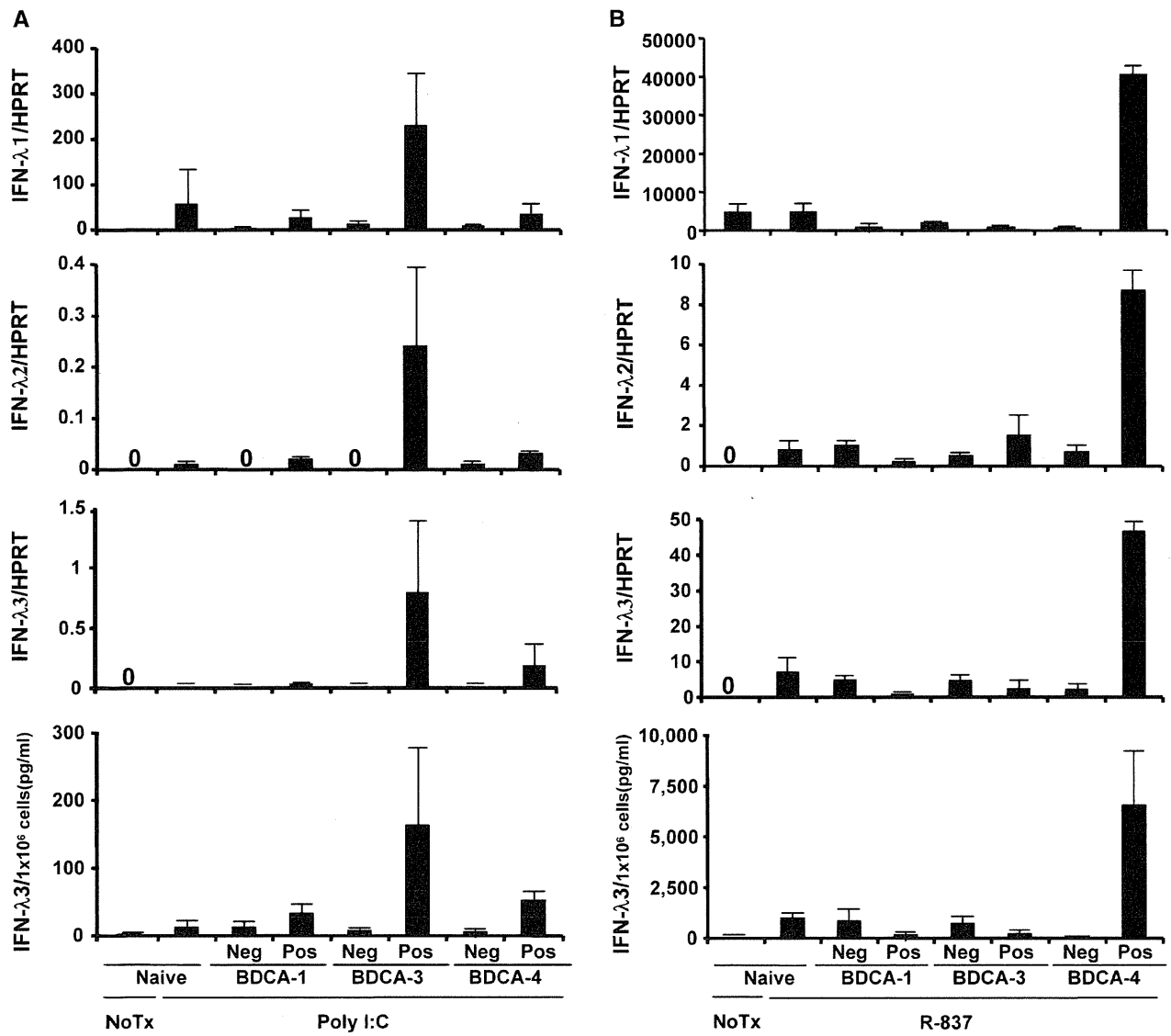

Fig. 1 IFN-λs were produced from different subsets of dendritic cells (DCs) when stimulated with different TLR agonists. BDCA-3^+ or BDCA-4^+DCs was negatively or positively selected using peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 5$). PBMC or DCs were stimulated with IFN-α, following poly I:C (**a**) or R-837 (**b**). The mRNA and the protein levels of IFN-λs were determined by real-time PCR and CLEIA, respectively. *Neg* negative selection, *Pos* positive selection of each DCs

BDCA-3$^+$ or 4$^+$DCs are very minor subsets in peripheral blood (0.03, 0.5 %, respectively) [21]. Therefore, we examined if a small amount of PBMC, using 2–3 ml of whole blood from healthy volunteers with negative anti-HCV (Supplementary Table 1), still induced detectable levels of IFN-λs. We confirmed that, even with a small amount of PBMC, detectable levels of IFN-λs were induced by R-837 and the levels of IFN-λ3 were different between *IL28B* genotypes (Fig. 2a, b). Therefore, whole PBMC from healthy volunteers with the TT genotype were

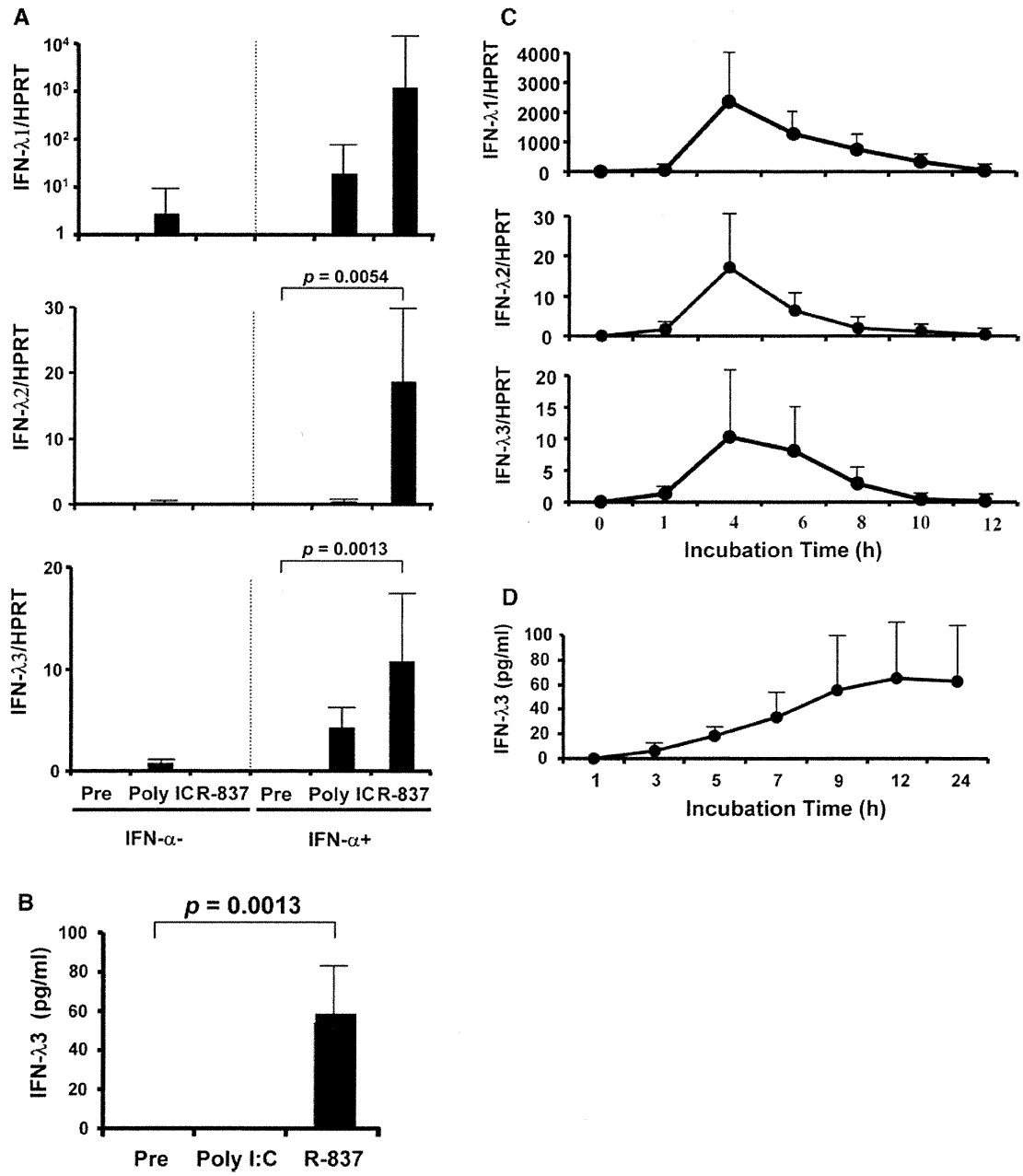

Fig. 2 Ex vivo induction of IFN-λs in PBMC from healthy volunteers. **a** mRNA expression levels of IFN-λs by real-time quantitative PCR. After pre-treatment with or without 100 U/ml of IFN-α for 16 h, 100,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (a TLR3 agonist) or 5 μg/ml of R-837 (a TLR7 agonist). After stimulation with TRL-agonists for 4 h, the PBMC were harvested. **b** Protein levels of IFN-λs. After pre-treatment with 100 U/ml of IFN-α, 200,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (a TLR3 agonist) or 5 μg/ml of R-837 (a TLR7 agonist), and the supernatant was harvested 24 h after stimulation with TLR agonists. **c** Kinetics of IFN-λs mRNA levels. After pre-incubation with IFN-α for 16 h, PBMC was stimulated with 5 μg/ml of R-837. Real-time quantitative PCR was conducted at each time point. **d** CLEIA results for IFN-λ3 protein in the supernatant at each time point