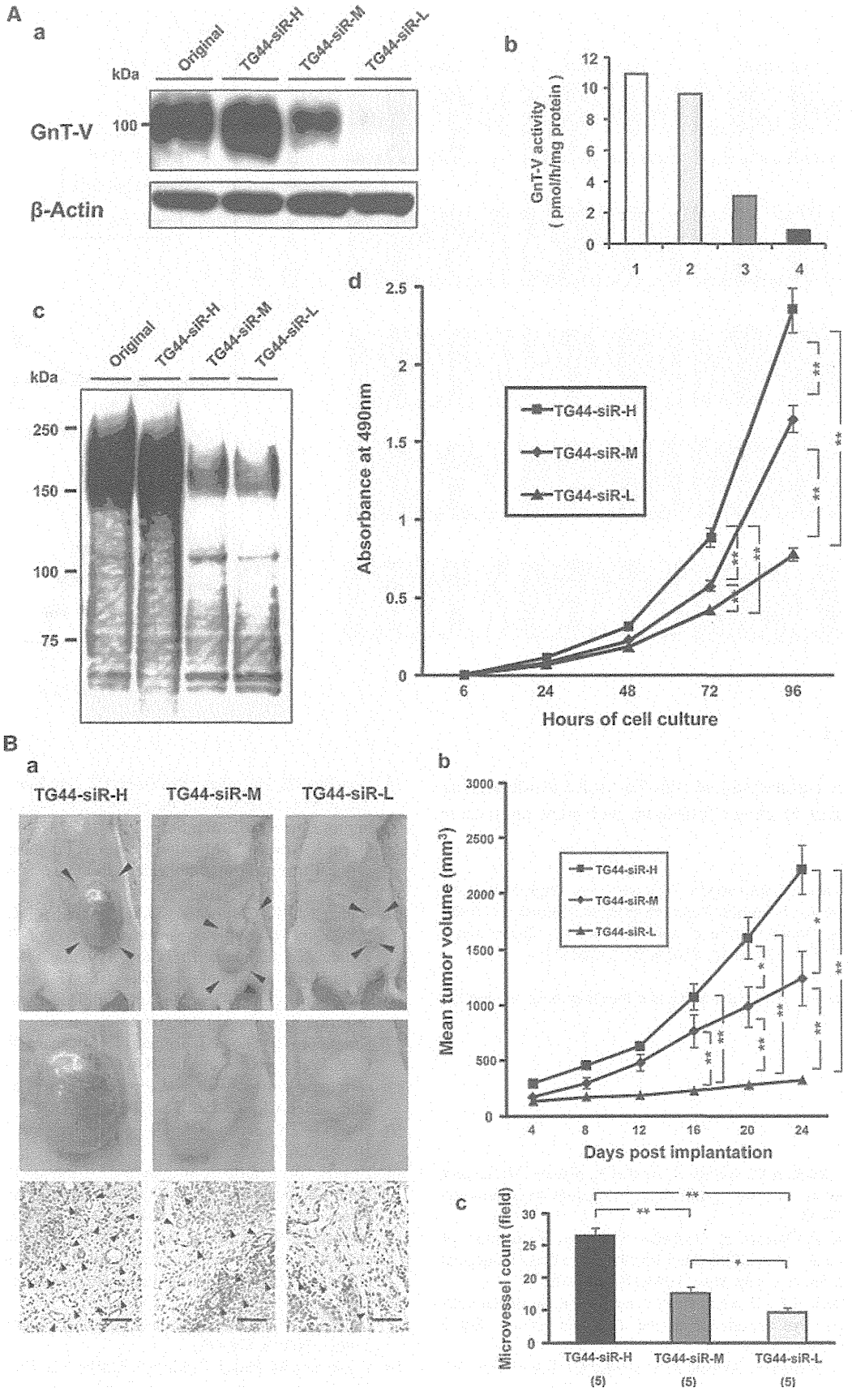
specificity of i-extension enzyme and 1–4 galactosyltransferase I [41]. This structure is preferentially fucosylated to form sialyl Lewis X, a ligand for selectin in vascular endothelial cells. The level of poly *N*-acetyllactosamine is increased in highly metastatic colon carcinoma cells [42] and that of sialyl Lewis X expression is correlated with poor survival in human colon cancer patients [43, 44]. From these points, GnT-V may induce tumor metastasis through the formation of sialyl Lewis X on the tumor cell surface.

However, in several types of cancers, GnT-V expression correlated inversely with the patient survival outcome [20, 21]. It has been postulated that involvement of GnT-V in tumor biology depends on whether its original organ tissues consist of β1-6 branching of *N*-linked oligosaccharides [20]. When cancers originate from tissues expressing no expression of β1-6 branching oligosaccharides, the induction of GnT-V expression is associated with malignant potential of the cancer cells [18]. On the other hand, when cancers originate from tissues expressing β1-6 branching oligosaccharides, the presence of GnT-V expression is an indicator of good prognosis [20]. Therefore, it should be stressed that the biological significance of GnT-V among cancers is uneven depending on the expression status of GnT-V and β1-6 branching oligosaccharides in original organ tissues. In this study, since normal gallbladder epithelia show no or only trace expression of GnT-V (Table 1), it is likely that the induction of GnT-V expression in gallbladder carcinoma is associated with the observed malignant behaviors of the cancer cells (Tables 4, 5).

Moreover, GnT-V functions as an angiogenesis inducer that has a completely different function from the original function of glycosyltransferase [17]. A secreted type of GnT-V protein itself promotes angiogenesis in vitro and in vivo [30, 45]. These findings provide novel information on the interrelationship among GnT-V, tumor growth and metastasis. In this study, supporting the angiogenesis function of GnT-V, the microvessel density was significantly higher in the tissue specimens of pT_2 gallbladder carcinoma showing positive staining than in those showing negative staining (Fig. 1c). In the subcutaneous tumor models, tumor growth was significantly more rapid for the cells with higher GnT-V expression than for those with lower GnT-V expression. Similar to the clinical specimens, the density was significantly increased in the tumors consisting of cells with higher GnT-V expression than in those consisting of cells with lower GnT-V expression (Fig. 3b). Angiogenesis is the first regulatory step of tumor progression. As shown in the hepatometastatic tumor models (Fig. 3c), the progression of metastatic tumor cells in the liver was significantly more rapid in cells with higher GnT-V expression than in those with lower GnT-V expression. Based on the above-mentioned results, the malignant phenotype can, therefore, be blocked by a GnT-V inhibitor. Swainsonine, a potent GnT-V inhibitor, reduces tumor metastasis and tumor solid growth in mice [46, 47].

Fig. 3 Biological effects of GnT-V on gallbladder carcinoma cells in ▶ in vitro and in vivo tumor models. **A** Construction of GnT-V knockdown gallbladder carcinoma cells by siRNA, as described in the "Materials and Methods" section. Three clones (i.e., TG44-siR-H, TG44-siR-M, and TG44-siR-L, were selected based on the immunoblot analysis of GnT-V (**a**), and subjected to in vitro assays, namely, GnT-V enzyme activity measurement (**b**), the lectin blotting (**c**), and tumor cell growth assay (**d**). **B** Subcutaneous tumors were seeded in immunodeficient mice using TG44-siR-H, TG44-siR-M, and TG44-siR-L (**a**), as described in the "Materials and Methods" section. Each group consisted of 5 animals. Tumor size was measured every other 4 days (**b**), and tumor volume (mm^3) was calculated as 0.5× longest diameter × $width^2$. Tumor volumes are presented as mean ± SE of 5 mice for each group. Significant differences between the groups are indicated by $*P < 0.05$ and $**P < 0.01$. CD31 immunostaining in tumor tissue sections (**a**) and quantification of microvessel density (**c**). *Bars* 100 μm. *Columns* and *bars* represent means and SE of the microvessel densities in each group, respectively. Quantification of data of microvessel density in the subcutaneous tumor sections was performed. A tumor tissue section was prepared from each of the 5 mice in the TG44-siR-H-, TG44-siR-M-, and TG44-siR-L-implanted groups. Five photographs were taken for each tissue section and then analyzed. Microvessels showing CD31 immunoreactivity were counted. Microvessel density for each of the 5 photographs of the tissue section was averaged, and the averaged densities of the above 3 groups (5 mice in each group) were compared. Significant differences between the groups are indicated by $*P < 0.05$ and $**P < 0.01$. **C** A persplenic hepatometastatic tumor model was developed in immunodeficient mice using TG44-siR-H, TG44-siR-L, and TG44-siR-M, all of which express luciferase, as described in the "Materials and Methods" section. Each group consisted of 5 mice. Sequential in vivo whole-body imaging of tumor progression was monitored over time using the IVIS imaging system (**a**). Panels depict 3 representative mice from each group. Time-course changes in the quantification of tumor bioluminescence were determined. Points, mean area of bioluminescence for live intact mice in each group ($n = 5$); *Bars* SE. Significant differences between groups are indicated by $**P < 0.01$ (**b**). The survival outcome of each group of mice with hepatometastatic tumors was assessed using Kaplan–Meier survival curves (**c**). The survival period of the TG44-siR-H group (median survival period, 34 days) was significantly shorter than that of the TG44-siR-M group (median survival period, 60 days) and that of the TG44-siR-L group (all mice were alive at the end point of the observation period). The differences were statistically significant according to the log-rank test ($*P < 0.05$ and $**P < 0.01$) (**c**)

In summary, the expression of GnT-V at the protein level is up-regulated in gallbladder carcinoma tissues and the immunohistochemical expression level of GnT-V in the subserosal layer of pT_2 gallbladder carcinoma is correlated with aggressiveness of the disease, such as the tendency to form distant recurrences, and with postsurgical survival. Moreover, in the in vitro and in vivo experiments using the gallbladder carcinoma cells, the expression levels of GnT-V in the cells were positively correlated with malignant behaviors, such as rapid cell growth, potent angiogenic capability, and potent metastatic potential. Taken together, the expression levels of GnT-V may serve as a unique biological feature associated with the malignant behavior


A
a
kDa
Original
TG44-siR-H
TG44-siR-M
TG44-siR-L
GnT-V
100
β-Actin
b
GnT-V activity
(pmol/h/mg protein)
12
10
8
6
4
2
0
1
2
3
4
c
kDa
Original
TG44-siR-H
TG44-siR-M
TG44-siR-L
250
150
100
75
d
Absorbance at 490nm
2.5
2
1.5
1
0.5
0
TG44-siR-H
TG44-siR-M
TG44-siR-L
6
24
48
72
96
Hours of cell culture
**
B
a
TG44-siR-H
TG44-siR-M
TG44-siR-L
b
Mean tumor volume (mm3)
3000
2500
2000
1500
1000
500
0
TG44-siR-H
TG44-siR-M
TG44-siR-L
4
8
12
16
20
24
Days post implantation
*
**
c
Microvessel count (field)
30
25
20
10
0
TG44-siR-H
(5)
TG44-siR-M
(5)
TG44-siR-L
(5)

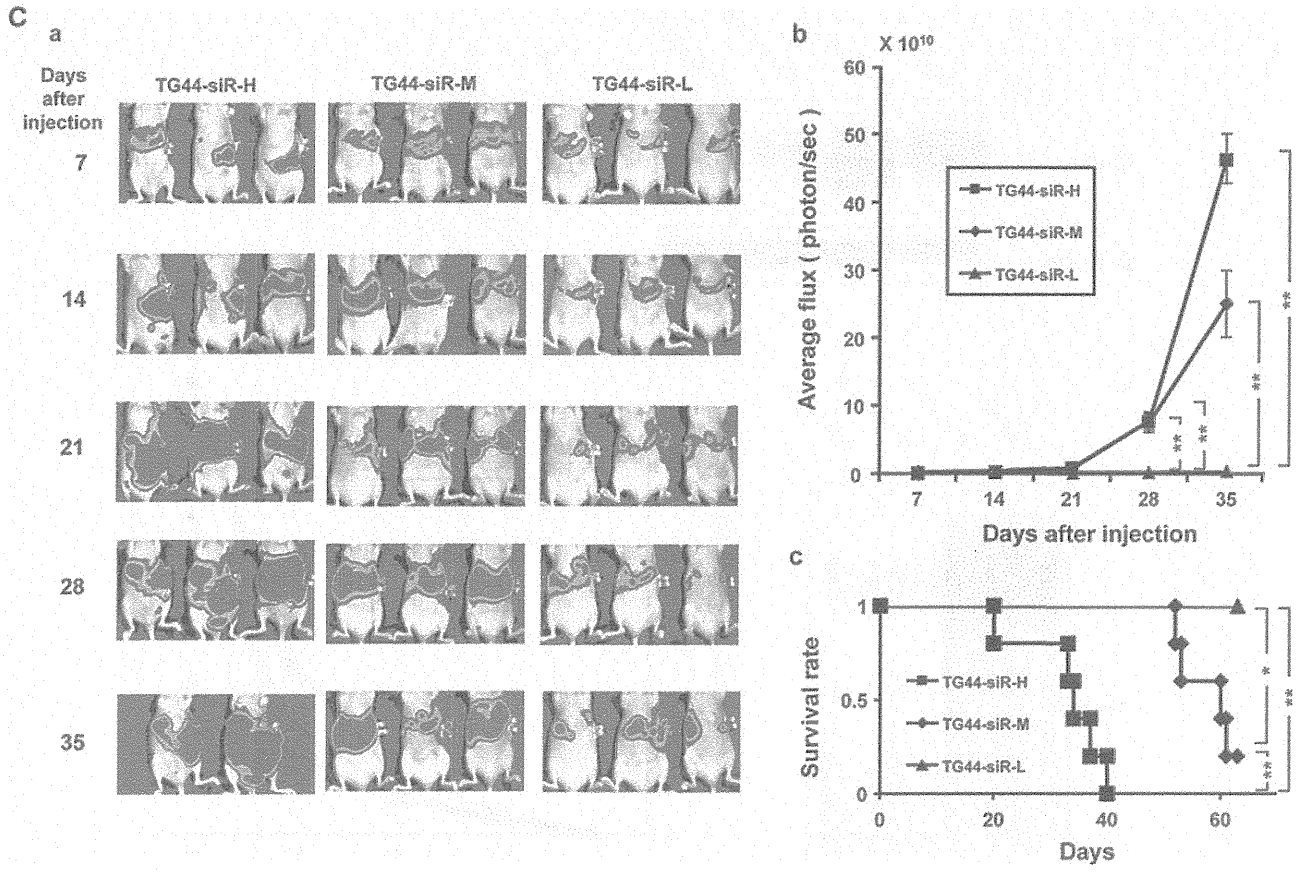


Fig. 3 continued

of gallbladder carcinoma and may be useful in identifying patients in need of closer follow-up and more aggressive treatment.

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Conflict of interest The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Serum Mac-2 binding protein levels as a novel diagnostic biomarker for prediction of disease severity and nonalcoholic steatohepatitis

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Purpose: Mac-2 binding protein (Mac-2bp) is one of the major fucosylated glycoproteins, which we identified with glycol-proteomic analyses. We previously reported that fucosylated glycoproteins are secreted into bile, but scarcely secreted into sera in normal liver and hypothesized that the fucosylation-based sorting machinery would be disrupted in ballooning hepatocytes due to the loss of cellular polarity. In the present study, we investigated the availability of Mac-2bp for differential diagnosis of nonalcoholic steatohepatitis (NASH) from nonalcoholic fatty liver disease (NAFLD) as a biomarker.

Experimental design: Serum Mac-2bp levels were determined with our developed ELISA kit. Our cohort of 127 patients with NAFLD had liver biopsy to make a histological diagnosis of NASH and simple fatty liver.

Results: Mac-2bp levels were significantly elevated in NASH patients compared with non-NASH (simple steatosis) patients (2.132 ± 1.237 vs. 1.103 ± 0.500 μg/mL, $p < 0.01$). The area under the receiver-operating characteristic curve for predicting NASH by Mac-2bp was 0.816. Moreover, multivariate logistic regression analyses showed Mac-2bp levels could predict the fibrosis stage and the presence of ballooning hepatocytes in NAFLD patients.

Conclusions and clinical relevance: These results support the potential usefulness of measuring Mac-2bp levels in clinical practice as a biomarker for NASH.

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Abbreviations: **AAL**, *Aleuria aurantica* lectin; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **AUROC**, area under the receiver-operating characteristic (ROC) curve; **CK-18**, cytokeratin-18; **HCV**, hepatitis C virus; **IRI**, immunoreactive insulin; **Mac-2bp**, Mac-2 binding protein; **NAFLD**, nonalcoholic fatty liver disease; **NASH**, nonalcoholic steatohepatitis; **NPV**, negative predictive value; **PPV**, positive predictive value; **T-Chol**, total cholesterol

Keywords:
Ballooning hepatocyte / Liver fibrosis / Mac-2bp/90K / *N*-glycan / NASH/NAFLD

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1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is among the world's most common causes of chronic liver disease, and is a growing medical problem in industrialized countries [1]. A wide spectrum of histological changes has been observed in NAFLD, ranging from simple steatosis, which is generally nonprogressive, to progressive nonalcoholic steatohepatitis (NASH). A proportion of patients with NASH develop cirrhosis and hepatocellular carcinoma [2]. Approximately 30% of the general population has NAFLD and up to 5% of this population has NASH [3, 4]. Liver biopsy remains the gold standard for the NASH diagnosis and the grading liver disease severity in NAFLD patients [5, 6]. However, invasive liver biopsy is poorly suited as a diagnostic test for such a prevalent condition, and this, in turn, restricts therapeutic intervention. Therefore, the need for development and validation of a reproducible and noninvasive test that can accurately distinguish NASH from simple steatosis is urgent.

Recent findings in glycobiology include direct evidence of the involvement of oligosaccharide changes in human diseases [7]. Glycoproteomics has been in focus as a postgenomic research field for the identification of diagnostic markers [8, 9]. In particular, fucosylation, characterized by the addition of fucose to the glycans, is an important oligosaccharide modification involved in cancer and inflammation [10]. Various fucosylated proteins are reported to be biomarkers for human diseases [11, 12]. Furthermore, we also showed that fucosylation is a possible signal for the polarized secretion of fucosylated glycoproteins into bile ducts in the liver [13]. In normal hepatocytes, fucosylated glycoproteins produced in the liver would be secreted into bile, but not in the sera. While, ballooning hepatocytes are known as a typical pathological character of NASH and alcoholic hepatitis [14, 15]. In the ballooning hepatocytes, the microtubule cytoskeleton, which is essential for normal efficient vesicle transport in the hepatocyte, is destroyed [16]. Its destruction induces nascent protein retention and an increase in the diameter of hepatocyte. Collectively, the fucosylation-based sorting machinery would be disrupted in the ballooning hepatocyte, and fucosylated glycoproteins produced in the liver would be secreted into sera.

In the present study, we first identified Mac-2 (galectin-3) binding protein (Mac-2bp) as one of the major fucosylated glycoproteins secreted from a liver bile duct cancer cell line, HuccT-1. Second, we analyzed serum Mac-2bp levels using our developed ELISA in NAFLD patients. Our study demonstrated that serum Mac-2bp levels were significantly higher in NASH patients compared with non-NASH (simple steatosis) patients. Moreover, serum Mac-2bp levels were closely correlated with liver fibrosis severity and the presence of ballooning hepatocytes. Our findings suggest that serum Mac-2bp levels could be a useful biomarker for NASH diagnosis and prediction of disease severity.

2 Materials and methods

2.1 Ethical committee approval

The protocol and informed consent were approved by institutional review boards at Graduate School of Medicine, Osaka University. At the time of liver biopsy, written informed consent was obtained from all subjects, and this study was in accordance with the Helsinki Declaration.

2.2 Detection of fucosylated glycoproteins from culture media of HuccT-1 cell line

Bile duct cancer (cholangiocarcinoma) is one of the liver cancers whose histological character is associated with abundant fibrotic changes [17]. We supposed that bile duct cancer cell lines could produce fucosylated glycoproteins, which are linked to the progression of liver fibrosis. To identify the major secretary fucosylated glycoproteins, we used a liver bile duct cell line, HuccT-1. HuccT-1 cells were cultured in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS, 50 U/mL penicillin, and 100 μg/mL kanamycin at 37°C in 5% CO_2, and then cultured the same medium without 10% FBS. Approximately 500 mL of the serum-free conditioned media of HuccT-1 cells were condensed with ammonium sulfate precipitation. After removing excess salts with membrane hydrolysis, the medium was applied to *Aleuria aurantica* lectin (AAL) agarose column (J-OIL MILLS, Tokyo, Japan). Fucosylated proteins were eluted with 10 mM fucose and the eluted proteins were applied to 8% SDS-PAGE followed by CBB staining. To identify fucosylated proteins, the bands were subjected to N-terminal amino acid sequence.

Next, to show the presence of fucose residues in Mac-2bp produced from HuccT-1 cells media, we performed AAL blotting and Mac-2bp immunoblotting on immunoprecipitated Mac-2bp with or without Glycopeptidase F (Takara Bio, Inc., Shiga, Japan) digestion. Briefly, approximately 15 mL of serum-free conditioned media of HuccT-1 cells

were condensed using the centrifugal filter devices (Amicon Ultra-15, Millipore Corp., Billerica, MA), followed by immunoprecipitation using mouse monoclonal anti-human Mac-2bp antibody (67A1, see Supporting Information Fig. 1A) and protein A Sepharose (GE Healthcare, Piscataway, NJ). Immunoprecipitants were digested with or without Glycopepcidase F at 37°C overnight. Then, the samples were boiled and applied for lectin blotting analysis using AAL (J-OIL MILLS) or for immunoblotting analysis using rabbit polyclonal anti-human Mac-2bp antibody (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan).

2.3 NAFLD Patients and normal control (NC) subjects

Biopsy-proven 127 NAFLD patients were enrolled in this study. The NCs were sera from 23 healthy subjects who underwent a medical checkup. The sera remaining after the medical checkup were used after permission had been received from the subjects. The exclusion criteria from this study included a history of other hepatic diseases, a substance abuse-induced hepatic disorder, a history of alcohol abuse (defined as $\geq$20 g of alcohol daily).

2.4 Anthropometric and laboratory evaluation

Anthropometric variables were measured using a calibrated scale. Venous blood samples were obtained in the morning after overnight fasting. For all patients, biochemical variables were measured using a conventional automated analyzer (platelet counts, aspartate aminotransferase [AST], alanine aminotransferase [ALT], γ-glutamyl transpeptidase, albumin, total cholesterol [T-Chol], triglyceride, fasting blood glucose [FBS], immunoreactive insulin [IRI], ferritin, and hyaluronic acid).

2.5 Histological evaluation

All NAFLD patients in this study had received percutaneous liver needle biopsy. The biopsied liver samples were embedded in paraffin block according to standard procedure and stained with HE and Masson's trichrome stains. NASH was confirmed according to Matteoni's classification [18]. Patients whose liver biopsy specimens showed simple steatosis or steatosis with nonspecific inflammation were placed in the "non-NASH" cohort. Samples were also investigated and quantified according to NAFLD activity scoring [14]. Steatosis (0–3), lobular inflammation (0–2), and hepatocellular ballooning (0–2) were quantified. The individual parameters of fibrosis were scored independently according to the NASH clinical research network scoring system [14]. Advanced fibrosis was classified as a stage 2–4 disease.

2.6 Establishment of an ELISA system for quantification of Mac-2bp

We prepared full and partial length recombinant human Mac-2bps containing the FLAG tag at the C terminus expressed in HEK293 cells and purified it using ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich Co. LLC, Tokyo, Japan; Supporting Information Fig. 1A). We obtained mouse monoclonal antibodies using the purified Mac-2bp proteins as an immunogen and selected two clones, 8A2 and 67A1, which showed strong sandwich reactivity to Mac-2bp (Supporting Information Fig. 1B). To confirm whether these monoclonal antibodies specifically bind to human serum Mac-2bp, we performed Mac-2bp immunoblotting, using these two monoclonal antibodies (Supporting Information Fig. 2). Briefly, 20 μL of NASH patient sera were immunoprecipitated with rabbit polyclonal anti-human Mac-2bp antibody (Immuno-Biological Laboratories), and then immunoprecipitants were applied for immunoblotting analysis using the two mouse monoclonal antibodies (67A1, 8A2). We developed a sandwich Mac-2bp ELISA using a combination of 67A1 and 8A2. The capture antibody chosen was 8A2, which was eliminated Fc by pepsin digestion, and the detection antibody was HRP conjugated 67A1 mouse IgG. The ELISA assay system was finally designed as a kit (Immuno-Biological Laboratory Co., Ltd., Fujioka, Japan, code #27362).

The 96-well microtiter plates were coated by being filled with 100 μL of 100 mM carbonate buffer (pH 9.5) containing 0.5 μg of 8A2 F(ab')2 overnight at 4°C. The plates were then washed with PBS containing 0.1% Tween 20 (PBS-T), and blocked with 200 μL of 1% (w/v) BSA in PBS containing 0.05% NaN_3 overnight at 4°C. After washing twice with PBS-T, test samples and recombinant human Mac-2bp gradually diluted with 1% BSA in PBS-T per 100 μL as a standard was applied to the each well of the coated microtiter plate in duplicate, and incubated at 4°C for 1 h. After washing four times with PBS-T, 100 μL of HRP-conjugated mouse mAb (67A1) was applied to each well and incubated for 30 min at 4°C. Then, each well was washed five times with PBS-T, and 100 μL of tetramethylbenzidine (Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) solution was added to the wells as a substrate and incubated for 30 min in the dark at room temperature. By adding 100 μL of 1 M H_2SO_4, the reaction was terminated. The absorbance of the solution at 450 nm was determined in a microplate reader (E-Max; Molecular Devices, Sunnyvale, CA). For analysis, 4-parameter logistic curve fitting was used.

In order to assess intra- and interassay precision for ELISA, three quality controls (QCs) were established covering the high, mid, and low ranges of the standard curves. Intra-assay was determined by 24 repeated measurements of each QC sample in a plate. Interassay precision was determined by assessing each QC sample across six different plates with quintuple wells. To assess the recovery rate in the blood samples, different concentrations of recombinant Mac-2bp added samples were measured, and the recovery rate was validated

as the differentiation between the measured and the theoretical concentrations. The sensitivity of this kit was determined based on the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols (National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1 (1989), Villanova, PA: NCCLS). A standard curve using this ELISA kit was obtained using Mac-2bp recombinant proteins that were purified from the condition medium of HEK293 cells transfected with Mac2-bp expression vectors (Supporting Information Fig. 3).

2.7 ELISA for galectin-3 and caspase-cleaved cytokeratin-18 (CK-18)

Mac-2bp, also known as 90K, is a highly *N*-glycosylated, secreted protein, identified as a ligand of galectin-3 [19]. Galectin-3 has been reported to mediate cell–cell and cell–matrix interactions, apoptosis, cell proliferation, and angiogenesis [20–23]. A recent study demonstrated that galectin-3 deficiency protected mice against NASH progression [24]. Galectin-3 was measured using Human Galectin-3 Assay kit in accordance with the manufacturer's instructions (IBL, Gunma, Japan). Increasing evidence suggests that hepatocyte apoptosis is a key mediator of liver injury in NASH. On apoptosis of hepatocytes, cytokeratin (CK)-18, a major cytoplasmic intermediate filament protein in hepatocytes, is released into the blood. Thus, use of the M30 antigen (CK-18 fragments) levels has been proposed as a serum diagnostic marker of NASH [25, 26]. For the quantitative measurement of the caspase-generated neoepitope of CK-18, we used the M30-Apoptosense ELISA according to the instructions of the manufacturer (Peviva, Bromma, Sweden).

2.8 Statistical analysis

Statistical analysis was performed with JMP 9.0 software (SAS Institute, Inc., Cary, NC). Continuous data were presented as mean ± SD. Kruskal–Wallis tests, Wilcoxon tests, and chi-square tests were performed to assess the existence of any significant differences between groups. Spearman's correlation coefficient was used to assess the relationship between serum Mac-2bp and several factors of interest. The diagnostic performances of the markers were estimated using the receiver-operating characteristic (ROC) curves. The probabilities of sensitivity and specificity estimation were assessed for selected cutoff values and the area under receiver-operating characteristic curve (AUROC) was calculated for each index. We used the Youden index to determine the optimal cutoff points. Multivariate logistic regression analyses were conducted to identify parameters that significantly contribute to the diagnosis of NASH and the estimation of NASH disease severity. A p-value less than 0.05 denoted the presence a statistically significant difference.

3 Results

3.1 Identification of fucosylated glycoproteins from HuccT-1 cultured media

We identified three fucosylated glycoproteins from HuccT-1 cultured media (Fig. 1A). As a result from N-terminal amino acid sequence analyses, one of these proteins was identified as Mac-2bp (95 and 145 kDa), which has many potential *N*-glycan binding sites. Other two fucosylated proteins were identified to be thrombospondin2 and C3 component, respectively.

Next, to show the presence of fucose residues in Mac-2bp, we performed immunoprecipitation of Mac-2bp from the conditioned media of HuccT-1 cells, followed by AAL blotting. As shown in Fig. 1B, strong binding of AAL to immunoprecipitated Mac-2bp was detected and the density was reduced after Glycopeptidase F digestion. While the molecular weight of Mac2-bp was decreased with Glycopeptidase F treatment, the binding capacity to AAL was still observed, suggesting that Mac2-bp produced from HuccT-1 cells has *O*-glycans with fucosylation as reported previously [27].

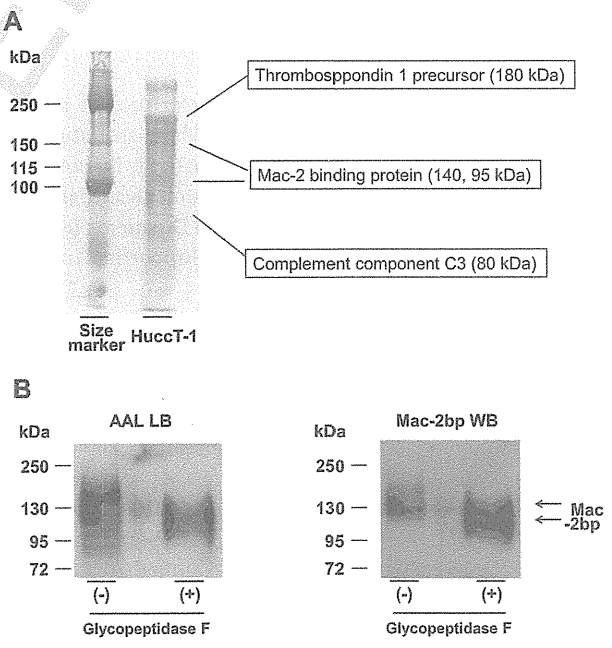

Figure 1. Mac-2 binding protein (Mac-2bp) was one of three major fucosylated proteins in the conditioned media of HuccT-1. (A) Srurm-free conditioned medium of HuccT1 cells was applied to *Aleuria aurantica* lectin (AAL) agarose column and binding proteins were subjected to SDS-PAGE followed by CBB staining. As a result from MS analyses, the bands were identified to be thrombospondin1, Mac-2bp, and complement C3. (B) To show the presence of fucose residues in Mac-2bp obtained from HuccT-1 cells media, we performed AAL blotting (left panel) and Mac-2bp immunoblotting (right panel) with (+) or without (−) Glycopeptidase F digestion after immunoprecipitation using anti-human Mac-2bp antibody. LB, lectin blotting, WB, Western blotting.

Table 1. Clinical and serological characteristics of the subjects Q8

Factor	All subjects ($n = 127$)	Non-NASH ($n = 18$)	NASH ($n = 109$)	p-value*
Age (y)	54.5 ± 12.8	47.5 ± 13.7	55.6 ± 12.4	<0.01
Sex (M/F)	68/59	13/5	55/54	0.086
BMI (kg/m^2)	27.3 ± 5.0	26.5 ± 3.5	27.5 ± 5.2	0.41
AST (U/L)	62.8 ± 39.1	44.7 ± 37.4	72.7 ± 50.0	<0.01
ALT (U/L)	96.0 ± 71.7	72.7 ± 50.1	99.8 ± 74.1	0.088
AST/ALT ratio	0.736 ± 0.284	0.639 ± 0.215	0.752 ± 0.291	0.12
GGT (U/L)	110.8 ± 117.0	152.1 ± 176.0	103.9 ± 103.6	0.62
T-Chol (mg/dL)	202.0 ± 38.9	208.1 ± 28.0	201.0 ± 40.4	0.41
TG (mg/dL)	152.5 ± 78.4	143.9 ± 62.0	153.9 ± 81.1	0.77
FBS (mg/dL)	112.4 ± 35.9	111.0 ± 36.2	112.7 ± 36.0	0.58
IRI (mU/mL)	13.7 ± 11.3	8.5 ± 3.9	14.4 ± 11.8	<0.05
Albumin (mg/dL)	4.14 ± 0.43	4.27 ± 0.31	4.12 ± 0.44	0.13
Ferritin (μg/dL)	335.8 ± 357.8	181.8 ± 144.9	362.5 ± 376.9	<0.05
Hyaluronic acid (g/dL)	71.9 ± 91.3	27.5 ± 17.9	79.0 ± 96.2	<0.01
Platelet count (× 10^4/μL)	20.0 ± 6.3	23.9 ± 5.5	19.5 ± 6.2	<0.01
Mac-2bp (μg/mL)	1.986 ± 1.214	1.103 ± 0.500	2.132 ± 1.237	<0.01
M30 (U/L)	825.8 ± 815.6	480.4 ± 556.0	882.8 ± 839.2	<0.01
Galectin-3 (ng/mL)	5.25 ± 3.57	4.84 ± 2.24	5.32 ± 3.75	0.95

Data are presented as the mean ± SD.
BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; T-Chol, total cholesterol; TG, triglyceride; FBS, fasting blood glucose; IRI, immunoreactive insulin.
p values correspond to the comparison between non-NASH and NASH group. Wilcoxon test for continuous factors and Pearson's chi-square test for categorical factors were used.

3.2 Characteristics of the subjects

The clinical and biochemical characteristics of individuals in this study are shown in Table 1. The distribution of Matteoni's classification among the 127 patients is shown in Supporting Information Table 1. The histological characteristics of liver biopsy specimens from NAFLD patients (non-NASH and NASH patients) are shown in Supporting Information Table 2.

3.3 Mac-2bp levels are significantly increased in NASH patients

Serum Mac-2bp levels were significantly increased in non-NASH patients compared with NC (1.103 ± 0.500 vs. 0.675 ± 0.271 μg/mL, $p < 0.01$; Fig. 2A). More importantly, the serum Mac-2bp levels in NASH patients exhibited greater increases than did those in non-NASH patients (2.132 ± 1.237 μg/mL, $p < 0.01$; Table 1, Fig. 2A). Serum M30 antigen levels were also significantly higher in NASH patients than in non-NASH patients (Table 1). We also compared the sensitivity and specificity of Mac-2bp with those of the M30 antigen for the discrimination of NASH using the ROC curve and the AUROC (Fig. 2B). The cutoff values were 1.288 μg/mL for Mac-2bp, and 310.6 U/L for the M30 antigen. The sensitivity of Mac-2bp was almost the same as that of the M30 antigen for the differentiation of NASH and non-NASH (74.3 vs. 78.9%). The specificity of Mac-2bp was higher than that of the M30 antigen (77.8 vs. 61.1%). The positive predictive value (PPV) and negative predictive value (NPV) of Mac-2bp were almost same as those of the M30 antigen (PPV: 95.3 vs. 92.5%, NPV: 33.3 vs. 32.4%). In addition, the AUROC of Mac-2bp was higher than that of the M30 antigen (0.816 vs. 0.725). There was no correlation between Mac-2bp and the M30 antigen levels in NAFLD patients (Fig. 2C, Supporting Information Table 3).

Mac-2bp concentrations had a significant positive association with NAFLD activity scoring ($r = 0.362$), but there was no significant correlation between Mac-2bp and hepatic steatosis scores ($r = -0.031$; Supporting Information Table 3, Fig. 3). Mac-2bp levels had strong positive correlations with hepatic inflammation ($r = 0.684$) and hepatocyte ballooning scores ($r = 0.707$). Above all, Mac-2bp levels significantly increased with the increase in the hepatocyte ballooning scores (Fig. 3C). In addition, Mac-2bp levels had a strong positive correlation with serum hyaluronic acid ($r = 0.427$) and the hepatic fibrosis scores ($r = 0.462$), but had a negative correlation with platelet count ($r = -0.202$).

3.4 Mac-2bp levels increased with fibrosis stage progression

Stepwise increase of Mac-2bp levels was observed with fibrosis stage progression (F0; 1.179 ± 0.526, F1; 1.489 ± 0.704, F2; 2.164 ± 1.548, F3 and 4; 2.468 ± 1.205 μg/mL, $p <$ 0.01; Supporting Information Fig. 4A). Mac-2bp levels in advanced fibrosis (F2–4) patients were significantly higher than those in nonfibrosis or mild fibrosis (F0–1) patients (Supporting Information Fig. 4B). The M30 antigen levels have been reported to predict fibrosis severity of NAFLD [26]. We

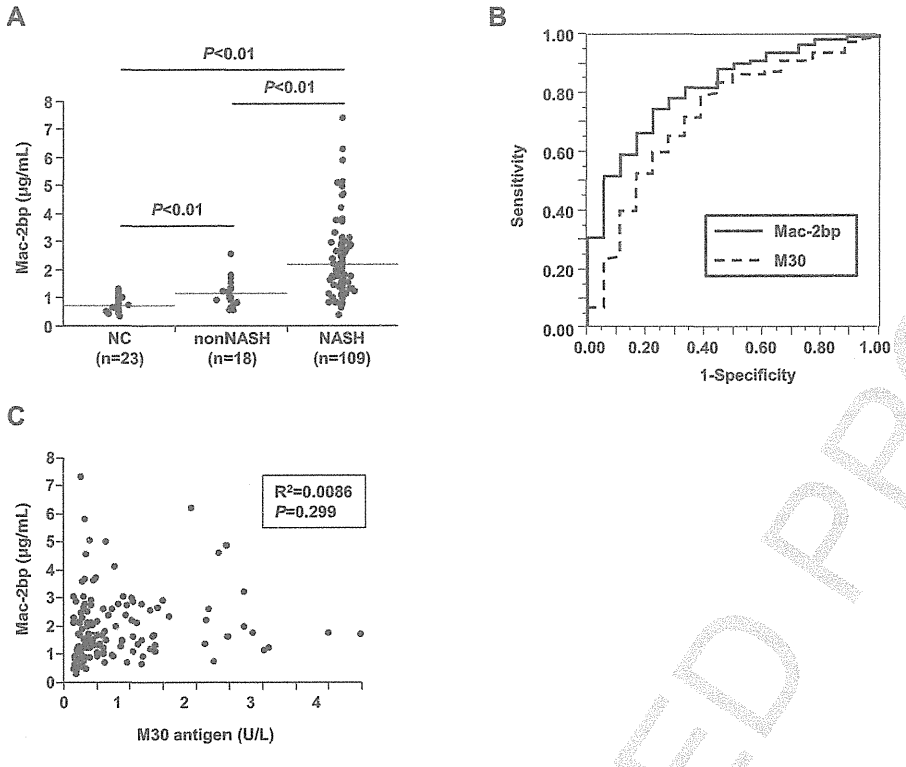

Figure 2. Serum Mac-2 binding protein (Mac-2bp) levels were significantly elevated in nonalcoholic steatohepatitis (NASH) patients. (A) Serum Mac-2bp levels in each group. Horizontal gray lines indicate the mean values of Mac-2bp in each group. NC, normal controls. (B) Receiver-operating characteristic (ROC) curves for Mac-2bp and the M30 antigen for the discrimination of NASH. (C) Correlation between Mac-2bp and the M30 antigen in nonalcoholic fatty liver disease (NAFLD) patients.

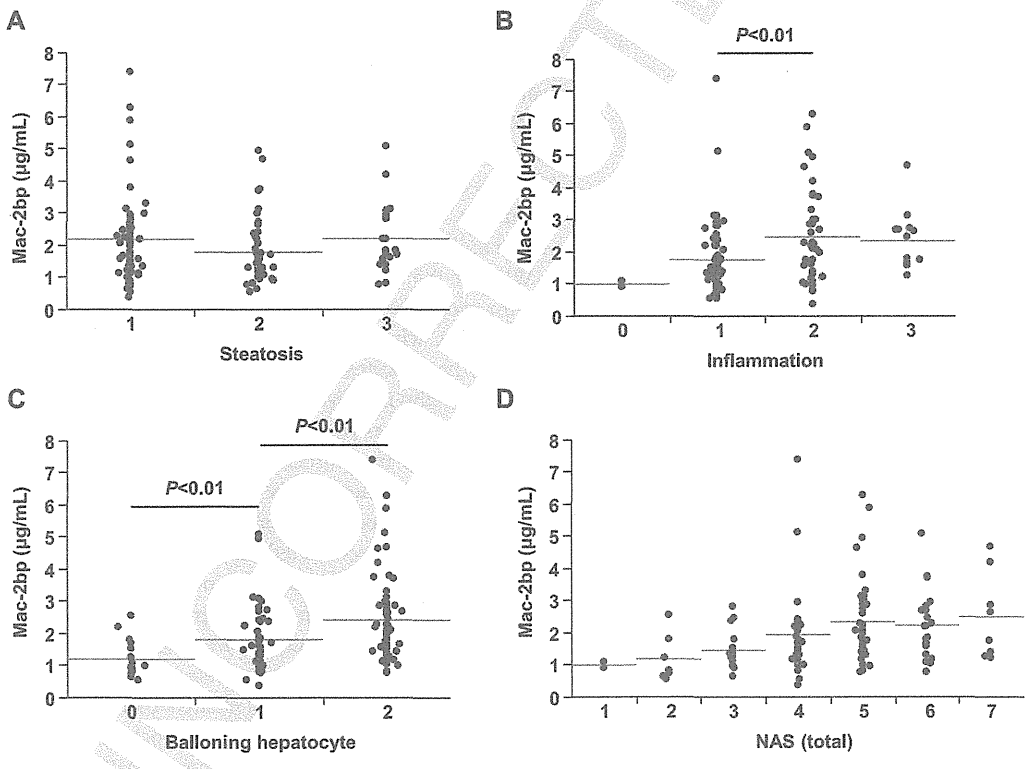

Figure 3. Serum Mac-2 binding protein (Mac-2bp) levels in each nonalcoholic fatty liver disease (NAFLD) activity scoring component. (A) Correlation between hepatic steatosis grade and serum Mac-2bp levels. (B) Correlation between lobular inflammation scores and serum Mac-2bp levels. (C) Correlation between hepatocyte ballooning scores and serum Mac-2bp levels. (D) Correlation between total NAS and serum Mac-2bp levels.

Table 2. Multiple logistic regression analysis of factors associated with NASH compared to non-NASH

Factor	Odds ratio	95% CI	*p*-value
Age	1.065	0.987–1.157	0.105
BMI	1.029	0.860–1.273	0.762
AST/ALT ratio	0.304	0.00715–16.178	0.53
T-Chol	0.998	0.971–1.025	0.896
TG	1.01	0.995–1.03	0.216
IRI	1.058	0.938–1.274	0.448
Albumin	0.922	0.0788–11.311	0.948
Ferritin	1.005	1–1.014	<0.05
Platelet count	0.945	0.801–1.105	0.471
Mac-2bp	4.161	1.0786–30.57	<0.05
M30	1	0.999–1.002	0.672

therefore compared the sensitivity and specificity of Mac-2bp with those of the M30 antigen for discrimination of advanced fibrosis using the ROC curve and the AUROC (Supporting Information Fig. 4C). The cutoff values were 1.491 μg/mL for Mac-2bp, and 636.1 U/L for the M30 antigen. The sensitivity of Mac-2bp was higher than that of the M30 antigen for the differentiation of fibrosis severity (76.6 vs. 50.7%), while the specificity was lower (68.0 vs. 78.0%). PPV of Mac-2bp was almost same as that of the M30 antigen (78.7 vs. 78.0%), while NPV of Mac-2bp was higher (65.4 vs. 50.7%). The AUROC of Mac-2bp was higher than that of the M30 antigen (0.743 vs. 0.655).

3.5 Mac-2bp levels are independent and significant determinants for diagnosis of both NASH and advanced fibrosis

Next, to investigate whether Mac-2bp levels independently correlate with the diagnosis of NASH and advanced fibrosis, we performed multivariate logistic regression analyses. Table 2 shows the findings for analyses of the non-NASH and NASH group. Serum Mac-2bp levels and ferritin were independent and significant determinants for the diagnosis of NASH.

Supporting Information Table 4 shows the findings for the multivariate logistic regression analysis of the stage 0–1 and stage 2–4 groups. We found that serum Mac-2bp levels were the only factor related to the fibrosis progression from stages 0–1 to stage 2–4.

4 Discussion

Distinguishing NASH from non-NASH patients and monitoring NASH disease progression are extremely important in the clinical management of NAFLD. Above all, a noninvasive and reliable approach is needed in the field. In the present report, we show that serum Mac-2bp was one of the major fucosylated glycoproteins in conditioned media of a cholangiocarcinoma cell line, and its levels were significantly elevated in NASH patients compared with non-NASH patients. Moreover, measurement of serum Mac-2bp concentrations was superior compared to measurement of the M30 antigen in distinguishing NASH patients from non-NASH patients and predicting fibrosis severity in NAFLD patients.

Mac-2bp is barely detectable in normal liver, but strong cytoplasmic staining has been observed in hepatocytes from hepatitis C virus (HCV) hepatitis patients [28]. Another research group identified serum Mac-2bp as a potential marker of fibrosis progression in HCV patients using proteome analysis [29]. These results prompted us to investigate whether or not serum levels of Mac-2bp can predict disease severity of chronic liver diseases including NASH. Since most Mac-2bp is produced from injured liver, fucosylated Mac-2bp might be correlated with total Mac2-bp. Therefore, we first assayed total Mac-2bplevels instead of fucosylated Mac-2bp levels.

Hepatocyte apoptosis is one of the major pathological features of NASH, and is recognized as a contributor to liver fibrosis [25]. The M30 antigen, caspase-3-generated CK-18 fragments, has recently been identified as useful as a single biomarker for NASH diagnosis [25, 26]. Indeed, our study also demonstrated that serum M30 antigen levels were significantly elevated in NASH patients compared with non-NASH patients. AST/ALT ratio, IRI, and serum ferritin also have been tested in NAFLD subjects [30–33]. Multivariate logistic regression analysis in our study demonstrated that serum Mac-2bp is a significant determinant of NASH after adjustment for the M30 antigen, AST/ALT ratio, IRI, and ferritin levels. At present, various scoring systems for distinguishing NASH from non-NASH and measuring NAFLD disease severity are available as noninvasive approaches [31, 34–36], yet, there are few single biomarkers which have gained clinical validity. Our study clearly showed that serum Mac-2bp is a novel and useful single biomarker for NASH diagnosis.

Why are the serum Mac-2bp levels so strongly correlated with diagnosis for NASH? Mac-2bp was barely detectable in normal liver, while strong cytoplasmic expression was observed in hepatocytes in chronic hepatitis type C patients [28, 37]. Mac-2bp is a glycoprotein, which has seven potential *N*-glycosylation sites [19, 38], and fucosylation of *N*-glycan in Mac-2bp is seen in colorectal cancer cell lines [39]. The fucosylation is characterized by the addition of fucose to the glycans. We reported previously that many glycoproteins in bile were strongly fucosylated compared to serum glycoproteins, and suggested that fucosylation may be a possible signal for polarized secretion of glycoproteins into bile in the liver [13]. Indeed, we recently showed that fucosylated alpha-fetoprotein is more selectively secreted into bile [40]. In the present study, we found a strong positive correlation between serum Mac-2bp levels and ballooning hepatocyte scores. Ballooning hepatocytes are known as a typical pathological character of NASH [14, 15]. In the ballooning hepatocyte, the microtubule cytoskeleton, which is essential for normal efficient vesicle transport in the hepatocyte, is destroyed [16]. Its destruction induces nascent protein retention and an increase

Clinical Relevance

Nonalcoholic fatty liver disease (NAFLD) is a growing medical problem around the world. Among NAFLD patients, those with nonalcoholic steatohepatitis (NASH) can develop cirrhosis and hepatocellular carcinoma. The ability to distinguish NASH from simple steatosis would be of great clinical significance. Ballooning hepatocytes are characteristic of typical pathological NASH. In the ballooning hepatocytes, loss of cellular polarity followed by abnormal secretion of hepatic glycoproteins is assumed due to their destroyed cytoskeleton. We previously reported that fucosylated glycoproteins are secreted into bile, but scarcely secreted into sera in normal liver. Therefore, we hypothesized that the fucosylation-based sorting machinery would be disrupted in ballooning hepatocytes, and serum fucosylated hepatic glycoproteins would increase in NASH patients. In this study, we investigated the availability of Mac-2 binding protein (Mac-2bp), which was one of the major fucosylated glycoproteins, as a serum biomarker for NASH and found that serum Mac-2bp levels can predict NAFLD disease severity. Mac-2bp levels determined by our developed ELISA kit were significantly elevated in NASH patients compared with non-NASH (simple steatosis) patients. Moreover, Mac-2bp levels could predict the fibrosis stage and the presence of ballooning hepatocytes in NAFLD patients. These results support the potential usefulness of measuring Mac-2bp levels in clinical practice as a biomarker for NASH.

in the diameter of hepatocyte. Collectively, the fucosylation-based sorting machinery would be disrupted in ballooning hepatocytes. Recently, we identified Mac-2bp as a fucosylated protein in the conditioned medium of cancer cell lines (data not shown). Before we investigated aberrant glycan changes in Mac-2bp of NASH patients, we measured the level of total Mac-2bp in this study. Consequently, serum Mac-2bp levels would be elevated in NASH patients compared to non-NASH patients. Further investigations should be conducted in future studies to analyze the fucosylation rate of Mac-2bp of NASH patients with MS and/or HPLC.

Using proteome analysis, Cheung et al. reported that serum Mac-2bp is a potential marker of fibrosis progression in HCV patients [29]. In our study, serum Mac-2bp levels were significantly and positively correlated with serum hyaluronic acid and fibrosis scores, but negatively correlated with platelet count. Surprisingly, multivariate logistic regression analysis showed that Mac-2bp was the only significant determinant for predicting advanced fibrosis in NAFLD patients. These results indicate that serum Mac-2bp is a unique fibrosis biomarker in NAFLD patients and useful for estimating the disease severity of NAFLD.

This study has several limitations. First, the proportion of non-NASH patients (14.2%) was small compared with that of NASH patients (85.8%). Second, patients were enrolled from Japanese hepatology centers that are specialized in NAFLD study, the referral bias potential could not be ruled out. In addition, some patient selection bias might have existed, because liver biopsy might have been taken into account for NAFLD patients who were likely to have NASH. Thus, the findings of this study might not represent NAFLD patients in the general population.

We conclude, despite these limitations, that serum Mac-2bp levels could distinguish NASH from non-NASH patients and estimate the disease severity of NAFLD patients with accuracy superior to that of the M30 antigen in our patients. Further investigation is needed using a larger number and wider spectrum of NAFLD patients.

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Dr. Kinoshita and Maruyama are employee of IBL. Other authors have declared no conflict of interest.

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PLOS ONE

Serum Fucosylated Haptoglobin as a Novel Diagnostic Biomarker for Predicting Hepatocyte Ballooning and Nonalcoholic Steatohepatitis

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a growing medical problem around the world. NAFLD patients with nonalcoholic steatohepatitis (NASH) can develop cirrhosis and hepatocellular carcinoma. The ability to distinguish NASH from simple steatosis would be of great clinical significance. Ballooning hepatocytes are characteristic of typical pathological NASH; here, the polarized secretion of proteins is disrupted due to destruction of the cytoskeleton. We previously reported that fucosylated glycoproteins are secreted into bile, but not into sera in normal liver. Therefore, we hypothesized that the fucosylation-based sorting machinery would be disrupted in ballooning hepatocytes, and serum fucosylated glycoproteins would increase in NASH patients. To confirm our hypothesis, we evaluated serum fucosylated haptoglobin (Fuc-Hpt) levels in biopsy-proven NAFLD patients (n = 126) using a lectin-antibody ELISA kit. Fuc-Hpt levels were significantly increased in NASH patients compared with non-NASH (NAFLD patients without NASH) patients. Interestingly, Fuc-Hpt levels showed a significant stepwise increase with increasing hepatocyte ballooning scores. Multiple logistic regression analysis showed that Fuc-Hpt levels were independent and significant determinants of the presence of ballooning hepatocytes. Moreover, Fuc-Hpt levels were useful in monitoring liver fibrosis staging. Next, to investigate the significance of serum Fuc-Hpt in a larger population, we measured Fuc-Hpt levels in ultrasound-diagnosed NAFLD subjects (n = 870) who received a medical health checkup. To evaluate NAFLD disease severity, we used the FIB-4 index (based on age, serum AST and ALT levels, and platelet counts). Fuc-Hpt levels increased stepwise with increasing FIB-4 index.

Conclusion: Measurement of serum Fuc-Hpt levels can distinguish NASH from non-NASH patients, and predict the presence of ballooning hepatocytes in NAFLD patients with sufficient accuracy. These results support the potential usefulness of measuring Fuc-Hpt levels in clinical practice.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is among the most common causes of chronic liver disease in the world and is a growing medical problem in industrialized countries [1]. A wide spectrum of histological changes has been observed in NAFLD, ranging from simple steatosis (which is generally non-progressive) to nonalcoholic steatohepatitis (NASH), and a proportion of patients with NASH develop cirrhosis and hepatocellular carcinoma (HCC) [2]. Approximately 30% of the general population has NAFLD and up to 5% of the population has NASH [3–5]. Liver biopsy remains the gold standard for diagnosing NASH and grading the severity of liver damage [6,7]. However, invasive liver biopsy is poorly suited as a diagnostic test for such a prevalent condition, and this in turn restricts therapeutic intervention. Moreover, biopsy itself carries significant limitations such as pain, risk of severe complications, sampling error [8], cost [9], and patient unwillingness to undergo invasive testing. Therefore, the need for development and validation of a reproducible and noninvasive test that can accurately distinguish NASH from simple steatosis is urgent.

Recent findings in glycobiology include direct evidence of the involvement of oligosaccharide changes in human diseases [10].

Glycoproteomics has been in focus as a post-genomic research field for the identification of diagnostic markers [11,12]. In particular, fucosylation, characterized by the addition of fucose to the glycans, is an important oligosaccharide modification involved in cancer and inflammation [13]. Various fucosylated proteins are reported to be biomarkers for human diseases [14–16]. We previously reported that fucosylated-haptoglobin (Fuc-Hpt) is a novel marker for patients with pancreatic cancer and colon cancer [16–18]. Haptoglobin is an acute phase protein mainly produced in the liver, and we have previously reported that interleukin-6 (IL-6), a typical inflammatory cytokine, upregulated fucosylation regulatory genes [19]. Furthermore, we also showed that fucosylation is a possible signal for the polarized secretion of fucosylated glycoproteins into bile ducts in the liver [20]. In normal hepatocytes, Fuc-Hpt produced in the liver would be secreted into bile, and not in the sera. On the other hand, ballooning hepatocytes are known as a typical pathological characteristic of NASH and alcoholic hepatitis [21–23]. In ballooning hepatocytes, the microtubule cytoskeleton, which is essential for normal efficient vesicle transport in the hepatocyte, is destroyed [24]. Its destruction induces nascent protein retention and an increase in the diameter of the hepatocyte. Collectively, the fucosylation-based sorting machinery would be disrupted in the ballooning hepatocyte, and Fuc-Hpt produced in the liver would be secreted into sera. Indeed, serum Fuc-Hpt levels assessed by western blotting were elevated in patients with alcoholic liver diseases [25]. In this study, we hypothesized that serum fucosylated glycoproteins levels would be elevated in NASH patients.

In the present study, to confirm our hypothesis, we analyzed serum Fuc-Hpt levels using a lectin antibody enzyme-linked immunosorbent assay (ELISA) in biopsy-proven NAFLD patients and subjects who underwent health check-ups. Our study demonstrated that serum Fuc-Hpt levels were significantly higher in NASH patients compared with non-NASH (NAFLD patients without NASH) patients. Moreover, serum Fuc-Hpt levels were closely correlated with the presence of ballooning hepatocytes. Our findings suggest that serum Fuc-Hpt levels could be a novel and useful biomarker for noninvasive NASH diagnosis.

Patients and Methods

Ethics Statement

The protocol and informed consent were approved by institutional review boards at Osaka University Graduate School of Medicine. Written informed consent was obtained from all patients at the time of liver biopsy or the medical health check-up, and the study was conducted in accordance with the Helsinki Declaration.

Biopsy-proven NAFLD Patients Study

A total of 126 patients with NAFLD confirmed by liver biopsy between 2008 and 2012 were enrolled from the following institutes: Osaka University Hospital, Ikeda Municipal Hospital, Otemae Hospital, and Osaka City University Hospital. The normal controls (NC) were sera from 24 healthy subjects who underwent a health checkup. The sera remaining after the medical checkup were used after permission had been received from the subjects. Sera from the liver biopsy-proven NAFLD patients were kept frozen at −80°C until used. The histological criterion used for the diagnosis of NAFLD was the presence of macrovesicular fatty changes in the hepatocytes, with displacement of the nuclei to the cell edge [26]. The criteria for exclusion from this study were as described below in the health checkup study.

Chronic Hepatitis Type C Patients

To compare the serum Fuc-Hpt levels with NAFLD patients, 29 patients with chronic hepatitis type C (CHC) were enrolled. These patients were collected at Osaka University Hospital from 2009 to 2012. Sera were taken at the time of liver biopsy, and histological evaluation was performed.

Medical Health Check-up Study

Among 1,500 Japanese adult subjects (1,131 males, 369 females) who underwent health checkups at aMs New Otani Clinic (Osaka, Japan) from 2008 to 2009, 1,044 subjects (797 male, 247 female) were recruited into this study. The criteria for exclusion from this study included a history of hepatic disease, such as chronic hepatitis C or concurrent active hepatitis B (seropositive for hepatitis B surface antigen), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, or hepatic injury caused by substance abuse, as well as a current or past history of consumption of more than 20 g of alcohol daily. Among the 1,044 subjects, 870 subjects (659 male, 211 female) were diagnosed with fatty liver, and 174 subjects (138 male, 36 female) were diagnosed without fatty liver by abdominal ultrasound. Serum from the subjects were collected at the health checkup and kept frozen at −80°C until used. The FIB-4 index (based on age, serum aspartate [AST] and alanine aminotransferase [ALT] levels, and platelet counts) was calculated for each of the subjects as previously reported (age × AST (U/L)/platelet count ($\times 10^9$/L)/$\sqrt{}$ALT (U/L)) [27,28]. The FIB-4 index has been demonstrated to be superior to other noninvasive markers of fibrosis in Japanese NAFLD patients [29]. The cutoff values proposed by Shah et al. [28] were adopted in this study (low cutoff point, 1.30; high cutoff point, 2.67).

Anthropometric and Laboratory Evaluation

Anthropometric variables (height and weight) were measured using a calibrated scale after requesting the patients to remove their shoes and any heavy clothing. Body mass index (BMI) was calculated as weight (kg) divided by the square of height in meters (m^2). Venous blood samples were obtained in the morning after the patients had fasted overnight for 12 hours. Laboratory evaluations for all patients included determination of platelet counts, and measurement of the serum levels of AST, ALT, γ-glutamyl transpeptidase (GGT), albumin, total cholesterol, triglyceride, fasting blood glucose, immunoreactive insulin (IRI), ferritin, and hyaluronic acid. All of the parameters were measured using standard techniques.

Histological Evaluation

All patients enrolled in this study had undergone percutaneous liver biopsy under ultrasound guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin and Masson's trichrome stains. Two hepatic pathologists (Y.K. and H.F.) who were blinded to the clinical data reviewed the liver biopsy specimens. Adequate liver samples were defined as >1.5 cm long and/or having more than six portal tracts. NASH was defined according to Matteoni's classification [30]. Patients whose liver biopsy specimens showed simple steatosis or steatosis with non-specific inflammation were placed in the "non-NASH" cohort. Samples were also investigated and quantified according to NAFLD activity scoring (NAS) [22]. Steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2) were quantified. The individual parameters of

fibrosis were scored independently according to the NASH Clinical Research Network scoring system [22]. Advanced fibrosis was classified as a stage 2–4 disease. In our study, decisions about each pathologic feature and the diagnosis of NASH were made by consensus between two hepatic pathologists [31]. Briefly, we diagnosed ballooning hepatocytes using HE staining as enlarged (>30 μm in diameter) with pale staining and a rarefied cytoplasma and rounded cell shape [32].

Lectin-antibody ELISA for Fuc-Hpt

The Fab fragment of antihuman haptoglobin IgG (Dako, Carpinteria, CA) was coated onto the bottom of a 96-well ELISA plate, because IgG has a fucosylated oligosaccharide in its Fc portion. Coated plates were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin for 1 hour, followed by washing with PBS containing 0.1% Tween 20 (PBS-T). A 50-μL aliquot of sera was placed into each well and incubated for 1 hour at room temperature. The plate was washed three times with PBS-T, using Immuno Wash (Bio-RAD Model 1517, Hercules, CA). To detect Fuc-Hpt, 1/1000 diluted biotinylated Aleuria aurantia lectin was placed into each well, followed by incubation at room temperature for 1 hour. After washing the plates three times with PBS-T, peroxidase-conjugated avidin was added to each well, followed by incubation at room temperature for 1 hour. After washing four times with PBS-T, tetramethylbenzidine was added to each well, followed by a 15-minute incubation for development. To stop the development, 1 N sulfuric acid was added to each well. A standard curve for Fuc-Hpt was obtained as previously described [19], using a Fuc-Hpt standard purchased from Takara Bio Inc. (Shiga, Japan).

ELISA for Haptoglobin, and Caspase-cleaved Cytokeratin-18 (M30 Antigen)

Haptoglobin was measured using the AssayMax Human Haptoglobulin ELISA kit (Assaypro, St. Charles, MO) in accordance with the manufacture's protocol. For the quantitative measurement of the caspase-generated neoepitope of cytokeratin-18, we used the M30-Apoptosense ELISA (Peviva, Bromma, Sweden) according to the instructions of the manufacturer.

Statistical Analysis

Statistical analysis was conducted using JMP 9.0 software (SAS Institute Inc Cary, NC). Continuous variables were expressed as mean ± standard deviation. Qualitative data were represented as numbers, with the percentages indicated in parentheses. Kruskal-Wallis tests and Wilcoxon tests were used to assess whether there were any significant differences in terms of continuous clinical or serological characteristics between groups. Chi-square tests were used for categorical factors. Spearman's correlation coefficient was used to estimate the association of serum Fuc-Hpt and several factors of interest. The diagnostic performances of the scoring systems were assessed by analyzing the receiver operating characteristic (ROC) curves. The probabilities of a true positive (sensitivity) and true negative (specificity) assessment were determined for selected cutoff values and the area under the receiver operating characteristic curve (AUROC) was calculated for each index. The Youden index was used to identify the optimal cutoff points. Multivariate logistic regression analyses were conducted to identify parameters that significantly contribute to the estimation of hepatocyte ballooning scores. Differences were considered statistically significant at $P<0.05$.

Results

Study of Biopsy-proven NAFLD Patients

At first, to investigate the significance of serum Fuc-Hpt levels in NAFLD patients, we measured serum Fuc-Hpt levels in biopsy-proven NAFLD patients (n = 126). The clinical and biochemical characteristics of individuals in this study are shown in Table 1. Significant differences were found between non-NASH and NASH groups in age ($P<0.01$), AST ($P<0.01$), IRI ($P<0.05$), ferritin ($P<0.05$), platelet count ($P<0.01$), hyaluronic acid ($P<0.01$), FIB-4 index ($P<0.01$), Fuc-Hpt ($P<0.01$), and M30 antigen ($P<0.05$). There were no significant differences between the two groups with respect to gender, BMI, ALT, AST/ALT ratio, GGT, total cholesterol, triglyceride, glucose, and albumin. The levels of serum haptoglobin also were not significantly different ($P = 0.9945$). The distribution of Matteoni's classification among the 126 patients is shown in Table S1. The histological characteristics of liver biopsy specimens from NAFLD patients (non-NASH and NASH) are shown in Table S2.

Next, we investigated the correlation coefficients of relationships between serum Fuc-Hpt levels and various parameters in the NAFLD patients (Table S3). Serum Fuc-Hpt levels showed significant and positive correlations with AST/ALT ratio ($r = 0.31$, $P<0.01$), hyaluronic acid ($r = 0.55$, $P<0.01$), FIB-4 index ($r = 0.40$, $P<0.01$), hepatocyte ballooning scores ($r = 0.41$, $P<0.01$), and fibrosis stage ($r = 0.41$, $P<0.01$). Fuc-Hpt levels had significant and negative correlations with platelet count ($r = -0.32$, $P<0.01$) and haptoglobin ($r = -0.27$, $P<0.01$). Fuc-Hpt levels had significant and positive correlations with NAS ($r = 0.28$, $P<0.01$) and inflammation scores ($r = 0.26$, $P<0.01$). There was no significant correlation between Fuc-Hpt levels and steatosis scores ($r = -0.16$, $P = 0.075$). Serum M30 antigen levels are known to be higher in NASH patients and their use in the diagnosis of NASH has been proposed [33,34]. There was no correlation between Fuc-Hpt and M30 antigen levels in NAFLD patients ($r = 0.056$, $P = 0.54$) (Table S3).

Fuc-Hpt Levels are Significantly Increased in NASH Patients

We examined whether serum Fuc-Hpt levels were useful for the diagnosis of NASH. There was no significant difference in Fuc-Hpt levels between normal control and non-NASH patients (167.8±211.0 vs. 111.1±202.1 U/mL, $P = 0.221$) (Table 1, Fig. 1A). Importantly, serum Fuc-Hpt levels in NASH patients exhibited greater increases than did those in non-NASH patients (655.9±1023.6 U/mL, $P<0.01$). In our study, serum M30 antigen levels were also significantly higher in NASH patients than in non-NASH patients (Table 1).

Among NAS, Fuc-Hpt levels showed a significant and stepwise increase with increased hepatocyte ballooning scores (Table 2, Fig. 2A), and fibrosis stage progression (F0; 22.7±42.0, F1; 353.6±813.0, F2; 512.7±771.0, F3 and 4; 899.9±1169.7 U/mL, $P<0.01$) (Fig. 3A). In addition, serum Fuc-Hpt levels had significant correlations with both typical serum fibrosis markers (platelet count, hyaluronic acid, FIB-4 index) and the histological fibrosis score (Table S3).

M30 antigen levels have been reported to correlate with NASH diagnosis, hepatocyte ballooning scores, and fibrosis severity in NAFLD patients [34]. We also compared the sensitivity and specificity of Fuc-Hpt with those of M30 antigen for the discrimination of NASH, the presence of ballooning hepatocytes (hepatocyte ballooning score 0 vs. 1 and 2), and the discrimination of advanced fibrosis (F0 & F1 vs. F2–4) using the ROC curve and the AUROC (Fig. 1B, 2B, 3B). All the AUROCs of Fuc-Hpt were

Table 1. Clinical and serological characteristics of the biopsy-proven NAFLD patients.

Factor	All subjects (n = 126)	nonNASH (n = 19)	NASH (n = 107)	*P* Value*
Age (yr)	54.4±12.8	46.9±13.6	55.7±12.3	<0.01
Gender (M/F)	70/56	13/6	57/50	0.2207
BMI (kg/m^2)	27.5±5.1	27.0±4.1	27.5±5.2	0.5646
AST (U/L)	62.9±39.3	44.2±36.5	66.3±39.0	<0.01
ALT (U/L)	95.8±72.0	71.9±48.8	100.1±74.7	0.0809
AST/ALT ratio	0.74±0.28	0.64±0.21	0.76±0.29	0.0752
GGT (U/L)	111.8±117.2	146.5±172.8	105.5±104.2	0.8392
Total cholesterol (mg/dL)	200.2±38.7	209.5±27.8	198.6±40.2	0.1690
Triglyceride (mg/dL)	152.8±78.7	145.4±60.6	154.2±81.8	0.8933
Glucose (mg/dL)	113.3±36.8	110.3±35.3	113.9±37.2	0.5067
IRI (mU/mL)	13.7±11.3	9.28±4.69	14.38±11.84	<0.05
Albumin (g/dL)	4.14±0.43	4.26±0.30	4.12±0.44	0.1187
Ferritin (μg/dL)	334.1±358.1	181.4±140.8	362.6±379.0	<0.05
Platelet count (x10^9/L)	200.3±64.3	248.8±67.1	191.7±60.1	<0.01
Hyaluronic acid (ng/dL)	73.0±91.9	26.5±17.8	81.1±97.1	<0.01
FIB-4 index	2.11±1.47	1.02±0.70	2.30±1.49	<0.01
Fuc-Hpt (U/mL)	573.8±965.7	111.1±202.1	655.9±1023.6	<0.01
M30 antigen (U/L)	811.3±780.8	573.6±511.1	854.3±814.6	<0.05
Haptoglobin (mg/dL)	89.3±29.5	89.4±29.9	89.3±29.5	0.9945

Data are presented as the mean ± SD.
Abbreviations: BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyl transpeptidase; IRI, immunoreactive insulin; Fuc-Hpt, fucosylated haptoglobin.
*P values correspond to the comparison between nonNASH and NASH group. Wilcoxon test for continuous factors and Pearson's Chi-square test for categorical factors were used.
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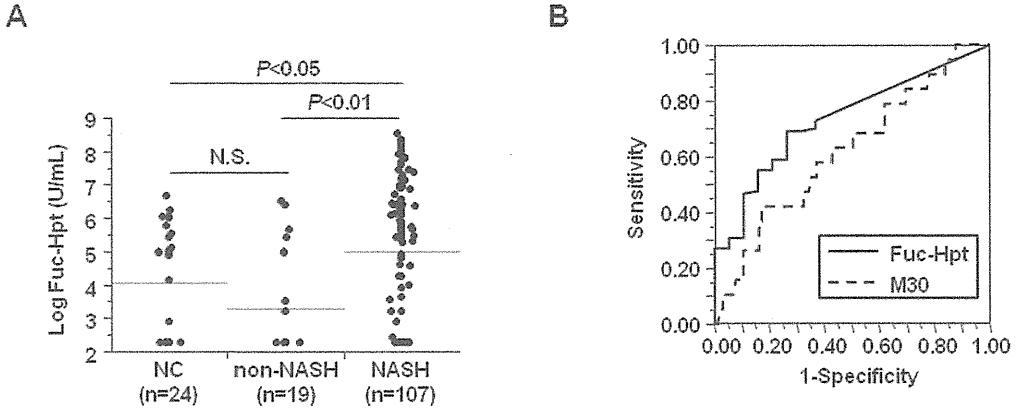
higher than that of the M30 antigen for the detection of NASH, ballooning hepatocyte presence, and advanced liver fibrosis (0.734 vs. 0.620, 0.759 vs. 0.607, and 0.724 vs. 0.636, respectively). The cutoff values for Fuc-Hpt were 36.1 U/mL (NASH diagnosis), 36.1 U/mL (ballooning hepatocyte discrimination), and 38.8 U/mL (fibrosis severity prediction). The cutoff values for the M30 antigen were 278.2 U/L (NASH diagnosis), 427.3 U/L (ballooning hepatocyte discrimination), and 770.1 U/L (fibrosis severity



Factor	AUROC	Sensitivity (%)	Specificity (%)	Accuracy (%)	cutoff value
Fuc-Hpt	0.734	69.16	73.68	69.84	36.1 (U/mL)
M30	0.620	42.10	82.86	75.40	278.2 (U/L)
Fuc-Hpt & M30	0.757	64.76	78.95	65.87	

Figure 1. Serum Fuc-Hpt levels were significantly elevated in biopsy-proven NASH patients. (A) Serum Fuc-Hpt levels in each group (normal controls, non-NASH patients, NASH patients). Horizontal grey lines indicate the mean values of Fuc-Hpt in each group. NC, normal controls. (B) ROC curves for Fuc-Hpt and the M30 antigen for the discrimination of NASH.
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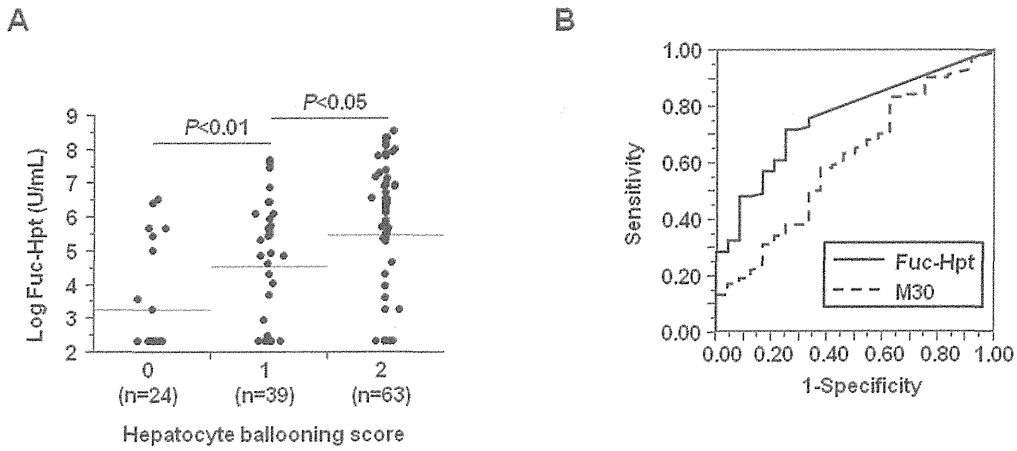

Factor	AUROC	Sensitivity (%)	Specificity (%)	Accuracy (%)	cutoff value
Fuc-Hpt	0.759	71.57	75.00	72.22	36.1 (U/mL)
M30	0.607	58.00	62.50	57.90	427.3 (U/L)
Fuc-Hpt & M30	0.780	67.00	79.17	69.25	

Figure 2. Correlation between serum Fuc-Hpt levels and hepatocyte ballooning scores in biopsy-proven NAFLD patients. (A) Serum Fuc-Hpt levels in each group classified by hepatocyte ballooning scores. (B) ROC curves for Fuc-Hpt and the M30 antigen for the presence of ballooning hepatocytes.
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prediction). The sensitivity of Fuc-Hpt was much higher than that of the M30 antigen for the differentiation of NASH and non-NASH (69.16 vs. 42.10%). However, the specificity of Fuc-Hpt was lower than that of the M30 antigen (73.68 vs. 82.86%). The accuracy of Fuc-Hpt was almost the same as that of the M30 antigen (69.84 vs. 75.40%) (Fig 1B). The sensitivity and specificity of Fuc-Hpt were higher than those of M30 antigen for the differentiation of the presence of hepatocyte ballooning (71.57 vs. 58.00%, 75.00 vs. 62.50%, respectively). The accuracy of Fuc-Hpt was also higher than that of M30 antigen (72.22 vs. 57.90%) (Fig. 2B). The sensitivity of Fuc-Hpt was much higher than that of M30 antigen for the differentiation of fibrosis severity (76.92 vs. 44.74%), while the specificity was lower (62.50 vs. 79.17%). The accuracy of Fuc-Hpt was higher than that of M30 antigen (71.43 vs. 57.14%) (Fig. 3B). The combination of Fuc-Hpt and M30 didn't enhance the significance of the detection of NASH, ballooning hepatocyte presence, and advanced liver fibrosis. Since these cut-off values for Fuc-Hpt were quite low, we adopted several cutoff values, and analyzed their sensitivity, specificity, and accuracy for the detection of NASH, ballooning hepatocyte presence, and advanced liver fibrosis (Table S4A, B, C).

Table 2. Clinical and serological characteristics of the biopsy-proven NAFLD patients classified by hepatocyte ballooning score.

	Hepatocyte ballooning score			
Variables	**0**	**1**	**2**	***P* value**
Number	24	39	63	
Age	46.1±12.7	52.9±12.2	58.4±11.7	<0.01
Gender (M/F)	13/11	27/12	29/34	0.0734
BMI	26.9±4.0	27.4±4.2	27.7±5.9	0.8026
AST	43.5±32.6	66.4±42.1	68.2±38.1	<0.01
ALT	78.0±47.0	106.7±84.8	95.9±70.9	0.4322
AST/ALT ratio	0.60±0.21	0.71±0.24	0.81±0.31	<0.01
GGT	133.4±157.4	102.0±81.4	109.6±119.2	0.9181
Total cholesterol	214.6±29.5	200.1±40.0	194.9±40.1	0.0633
Triglyceride	153.2±62.6	157.4±89.9	149.9±78.2	0.7822
Glucose	107.1±32.2	112.1±41.9	116.5±35.3	<0.05
IRI	9.45±4.97	10.97±5.62	17.20±14.55	<0.05
Albumin	4.27±0.28	4.22±0.45	4.04±0.44	<0.05
Ferritin	233.3±267.9	301.4±286.2	392.0±46.0	<0.05
Platelet count	250.2±61.5	193.5±55.5	185.6±61.8	<0.01
Hyaluronic acid	25.1±16.1	58.8±61.4	99.9±113.5	<0.01
FIB-4 index	0.97±0.64	2.07±1.42	2.57±1.50	<0.01
Fuc-Hpt	101.5±187.0	365.1±573.6	882.9±1206.2	<0.01
M30 antigen	586.0±493.6	720.3±690.1	954.3±895.8	0.17
Haptoglobin	88.1±29.1	93.5±30.7	87.3±29.1	0.5526

Data are presented as the mean ± SD.
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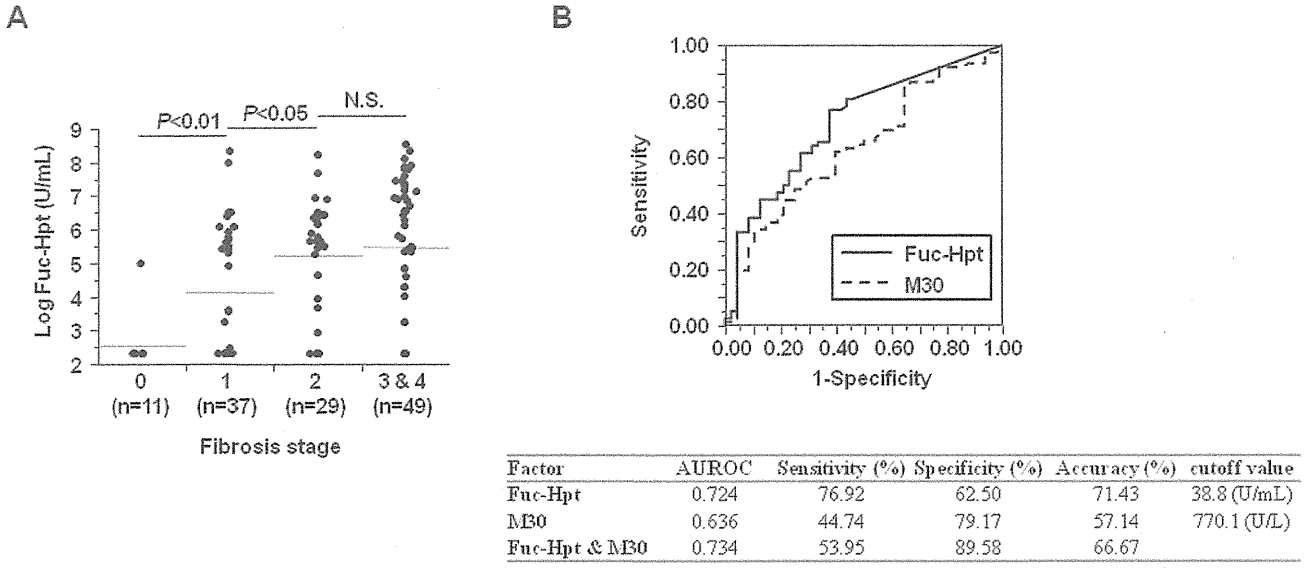
Next, we further evaluated the significance of serum Fuc-Hpt levels on the presence of ballooning hepatocytes by multiple logistic regression analysis (Table 3). Age, BMI, AST, GGT, total cholesterol, triglyceride, IRI, ferritin, platelet count, Fuc-Hpt, and M30 antigen were selected as variables based on multivariate analysis. We found that age, AST, platelet count, and Fuc-Hpt levels were independent and significant determinants for the discrimination of the presence of ballooning hepatocytes. M30 antigen levels were not a significant determinant in our study.

Comparison of Serum Fuc-Hpt Levels between NAFLD and Chronic Hepatitis Type C Patients

In order to investigate serum Fuc-Hpt levels in other liver disease, we compared serum Fuc-Hpt levels between NAFLD and CHC patients (Figure S1). The data indicated that serum Fuc-Hpt levels increased with the fibrosis stage progression in both NAFLD and CHC patients. In CHC patients, serum Fuc-Hpt levels increased especially in patients with advanced fibrosis (F3). Interestingly, NAFLD patients with hepatocyte ballooning score



Factor	AUROC	Sensitivity (%)	Specificity (%)	Accuracy (%)	cutoff value
Fuc-Hpt	0.724	76.92	62.50	71.43	38.8 (U/mL)
M30	0.636	44.74	79.17	57.14	770.1 (U/L)
Fuc-Hpt & M30	0.734	53.95	89.58	66.67	

Figure 3. Correlation between serum Fuc-Hpt levels and liver fibrosis stage in biopsy-proven NAFLD patients. (A) Serum Fuc-Hpt levels in each stage of liver fibrosis in NAFLD patients. (B) ROC curves for Fuc-Hpt and the M30 antigen for discrimination of advanced liver fibrosis in biopsy-proven NAFLD patients.
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2 showed high levels of serum Fuc-Hpt levels even in F1 and F2 stage patients. These results indicated that serum Fuc-Hpt levels cannot be used to distinguish NASH from CHC patients with advanced fibrosis, but could be a useful biomarker for the discrimination of NASH patients with early liver fibrosis. The presence of ballooning hepatocytes could elevate serum Fuc-Hpt levels in NASH patients.

Medical Health Check-up Study

To investigate the significance of serum Fuc-Hpt levels in a larger population, we measured serum Fuc-Hpt levels in NAFLD subjects (n = 870) who received health checkups. Table 4 lists the basic anthropometric data and the results of biochemical tests of all NAFLD subjects enrolled in the health checkup study. These subjects had not received liver biopsy, so we evaluated the hepatic disease severity of NAFLD subjects using the FIB-4 index in the health checkup study [27,28]. The FIB-4 index is based on age, serum AST and ALT levels, and platelet counts; these parameters are usually measured in health checkups in Japan. In addition, the FIB-4 index has been reported to be superior to other noninvasive markers of fibrosis in NAFLD patients [29]. The clinical and biochemical characteristics of individuals in the health checkup study classified by FIB-4 index categories (proposed by Shah et al. [28]) are shown in Table 5. Age ($P<0.01$), AST ($P<0.01$), AST/ALT ratio ($P<0.01$), GGT ($P<0.01$), and Fuc-Hpt ($P<0.01$) levels revealed significant stepwise elevation, while total cholesterol ($P<0.01$) and platelet count ($P<0.01$) showed significant stepwise decrease with progressively higher FIB-4 index categories. Multivariate logistic regression analysis indicated that serum Fuc-Hpt levels were independent and significant determinants for the prediction of F3 (by FIB-4 index) (odds ratio, 1.183; 95% CI 1.057–1.324; $P<0.01$) (Table S5). These results indicate that measurements of serum Fuc-Hpt levels could also predict NAFLD severity in a large population.

Table 3. Multiple logistic regression analysis of factors associated with hepatocyte ballooning score 1–2 compared to score 0.

		95% CI		
Factor	**Odds ratio**	**Lower**	**Upper**	***P* value**
Age	1.0737	1.0036	1.1596	<0.05
BMI	1.0343	0.8749	1.2624	0.7053
AST	1.0183	1.0001	1.0417	<0.05
GGT	0.9964	0.9892	1.0022	0.2326
Total cholesterol	0.9902	0.9670	1.0143	0.4102
Triglyceride	0.9991	0.9867	1.0128	0.8861
IRI	1.0755	0.9719	1.2688	0.1877
ferritin	0.9996	0.9969	1.0033	0.8133
Platelet count	0.9792	0.9600	0.9940	<0.01
Fuc-Hpt (per 100U/mL increase)	1.4196	1.0171	2.7146	<0.05
M30	1.0000	0.9989	1.0014	0.9386

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Discussion

Distinguishing NASH from non-NASH patients and monitoring NASH disease progression are extremely important in the clinical management of NAFLD. Above all, a noninvasive and reliable approach is needed in the field. In the present report, we show that serum Fuc-Hpt levels were significantly elevated in NASH patients compared with non-NASH patients. Ballooning hepatocytes are known as a typical pathological character of steatohepatitis including NASH [22,23,30]. The ability to detect the presence of ballooning hepatocytes is quite important in distinguishing NASH from simple steatosis. We show that serum Fuc-Hpt levels undergo a stepwise increase with increasing hepatocyte ballooning scores in biopsy-proven NAFLD patients in our study. In addition, Fuc-Hpt levels were significant and independent determinants for the discrimination of the presence of ballooning hepatocytes, even