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Clinicopathological Significance of Leucine-Rich α2-Glycoprotein-1 in Sera of Patients With Pancreatic Cancer

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Objectives: Leucine-rich α2-glycoprotein-1 (LRG-1) is an inflammatory protein. Serum LRG-1 levels can reportedly be used as a cancer biomarker for several types of carcinoma. In the present study, we investigated the clinical usefulness of serum LRG-1 levels as a biomarker of pancreatic cancer.

Methods: A total of 124 patients with pancreatic cancer, 35 patients with chronic pancreatitis (CP), and 144 healthy volunteers were enrolled in the study. Serum LRG-1 levels were assayed by enzyme-linked immunosorbent assay. Immunohistochemistry was used to examine LRG-1 expression in pancreatic cancer tissues.

Results: Serum LRG-1 levels were significantly increased in patients with pancreatic cancer compared with CP patients and healthy volunteers. The LRG-1 levels increased with progressive clinical stages of pancreatic cancer. Receiver operator curve analysis showed that a combination of carbohydrate antigen 19-9 and LRG-1 resulted in a higher area under the curve for the diagnosis of pancreatic cancer. Positive staining was observed in all cases of pancreatic cancer, but positive signal was scarcely detected in tissues from CP patients or normal surrounding tissue.

Conclusions: These results suggest that serum LRG-1 is a promising biomarker for pancreatic cancer.

Key Words: leucine-rich α2-glycoprotein-1, pancreatic cancer, biomarker

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Pancreatic cancer is the fourth leading cause of cancer-related death in the United States.[1] Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. In Japan, the mortality rate for PDAC has increased over the past decade, currently ranking fifth.[2] Pancreatic ductal adenocarcinoma has an extremely poor prognosis with a 5-year survival rate of less than 5%. Early detection of PDAC remains clinically challenging because of its asymptomatic nature, and surgery offers the only potential cure. However, the 5-year survival of PDAC patients after curative resection (surgery alone) is estimated to be 10% to 20%.[3] Curative surgery with adjuvant chemotherapy improves overall survival,[4] but the clinical outcome of pancreatic cancer has not been markedly improved. One strategy that could improve PDAC survival is to detect the cancer in its early clinical stages. Thus, there is an urgent need to develop biomarkers for the stratification of patients for current treatment modalities and the development of novel therapeutic strategies.

Molecules that are specifically overexpressed in tumors not only serve as useful diagnostic markers, but also as potential therapeutic targets.[5] The development of methods that are sensitive and specific enough to permit an early diagnosis of PDAC may facilitate the detection and subsequent treatment of this disease. However, an important factor adds another level of complexity to this already demanding task. A number of overlapping symptomatological features are known to link pancreatic adenocarcinoma to the inflammatory disease chronic pancreatitis (CP), often obscuring the distinction between these 2 pathological conditions.

Carcinoembryonic antigen, carbohydrate antigen 19-9 (CA19-9), and DUPAN-2 are the only serum biomarkers currently available for PDAC detection and have shown some utility as diagnostic adjuncts and prognostic markers in Japan.[6] However, these markers are not widely used in routine clinical practice in Europe and America due to low sensitivity, specificity, or reproducibility and therefore cannot be routinely used to diagnose PDAC. Although CA19-9 is not a specific marker, identifying characteristic CA19-9 carrier proteins may allow higher specificity (molecular proteomics). Although the detection of CA19-9 carrier proteins is established, it will be very difficult to detect the early stage of pancreatic cancer.[7] To establish a next-generation biomarker for pancreatic cancer, combination assays of several cancer biomarkers or a novel type of cancer biomarker involved in CP will be required.

Leucine-rich α2-glycoprotein-1 (LRG-1) was first identified in 1977 as an inflammatory protein in human serum.[8] Recent studies have demonstrated that serum LRG-1 can be used as a biomarker in several kinds of cancer, including ovarian, lung, biliary tract, and hepatocellular carcinoma related to hepatitis B virus infection.[9–13] Increased serum LRG-1 levels in patients with pancreatic cancer were first reported using multidimensional liquid chromatography followed by 2-dimensional difference gel electrophoresis in plasma proteomics.[14] The authors used Western blot to confirm the increase in serum LRG-1 patients with pancreatic cancer compared with healthy volunteers (HVs), and they showed that patients with CP tended to express lower serum levels of LRG-1 than patients with PDAC. However, they did not find significant differences between patients with CP and patients with PDAC due, at least in part, to the limited number of patients in the study. The study did not investigate the relationships between the marker and the clinical stages of pancreatic cancer, and no immunological study of LRG-1 in pancreatic cancer was performed. As

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LRG-1 has also been reported to be part of an acute inflammatory response in ulcerative colitis and acute appendicitis, LRG-1 may be increased as a result of inflammation.[15–18]

In the present study, we determined serum LRG-1 levels in patients with CP and PDAC and assessed the clinicopathological significance of the increase in LRG-1 in terms of clinical parameters and immunohistochemistry.

MATERIALS AND METHODS

Patients and Sample Collection

Peripheral blood samples from patients with pancreatic disease and apparently HVs were obtained from Ogaki Municipal Hospital, Japan Community Health Care Organization Osaka Hospital, and Osaka University Hospital. Written informed consent was obtained from all patients and HVs. The study was performed in accordance with the guidelines issued by the local institutional review board of Osaka University. Sera from 124 patients with PDAC and 35 patients with CP were evaluated. All blood sampling was done at the time of diagnosis (pretreatment). A total of 144 serum samples from HVs were used as controls. Detailed information on clinical background was available for 71 PDAC patients, 20 CP patients, and 91 HVs. The collection, processing, and storage of all blood samples were standardized as follows: blood samples were collected in a vacutainer tube, allowed to clot at room temperature for 30 minutes, and then centrifuged at ~2500 × g for 10 minutes. The serum was removed and immediately divided into 100-μL and 1-mL aliquots and stored at −80°C until use.

Formalin-fixed, paraffin-embedded tissue blocks from patients with PDAC were obtained from surgical cases at Osaka University Hospital in 2011 and 2012. Eleven PDAC, 1 CP, and 3 noncystic lesions of intraductal papillary mucinous neoplasm (IPMN; control for normal pancreas) were randomly selected.

Quantification of Serum LRG-1

Serum LRG-1 levels were determined using the human LRG assay kit (IBL, Fujioka, Japan) according to the manufacturer's protocol. Briefly, each serum sample was diluted (1:100) with buffer from the kit and the assays performed in triplicate. The LRG-1 levels were determined from a standard curve created using control samples. If the concentration of serum LRG-1 was less than 1.56 ng/mL or greater than 100 ng/mL, the dilution rate was changed.

Serum CA19-9 levels were measured in the hospital laboratory at the time of diagnosis.

Statistical Analysis

All statistical analyses were performed using JMP (version 9.0; SAS Institute Inc, Japan). Continuous data between clinical groups were compared by the Mann-Whitney *U* test for nonparametric data and the Student *t* test for normally distributed data. The Pearson product–moment correlation coefficient was used to examine associations between 2 continuous variables. Associations between categorical variables were examined using Fisher exact test. Normal cutoffs were defined for LRG-1 as the optimum point at which sensitivity and specificity was maximized. Receiver operating characteristic (ROC) curves were generated, and the areas under the curve (AUCs) were defined.

Immunohistochemical Staining

To analyze LRG-1 expression by immunohistochemistry, formalin-fixed, paraffin-embedded tissue blocks from patients with pancreatic cancer were deparaffinized in xylene and rinsed in a series of 100%, 95%, 90%, 80%, 70%, and 60% ethanol solutions. The antigen was recovered by incubating the slides in 10 mmol/L citrate buffer (pH 6.0) for 40 minutes in boiling water. To quench endogenous peroxidase activity, the slides were incubated with 0.3% H_2O_2 in methanol for 30 minutes and then rinsed extensively in phosphate-buffered saline. The slides were incubated with blocking serum for 20 minutes followed by a 1:400 dilution of rabbit anti-LRG monoclonal antibody (Cat# 13224-1-AP; ProteinTech Group, Chicago, Ill) overnight at 4°C. Endogenous biotin activity was blocked using an avidin D–biotin blocking solution before in situ localization of the antigen using a biotin-avidin antigen detection method (R.T.U. Vectastain kit, Vector Laboratories, Burlingame, Calif). After extensive washing in phosphate-buffered saline, sections were incubated in a diaminobenzidine solution (Stable DAB, Invitrogen, Carlsbad, Calif) and counterstained with a hematoxylin solution. The slides were then dehydrated by washing in ethanol and cleared in xylene. Cover slips were placed on the slides, which were evaluated for LRG-1 staining. The intensity of LRG-1 staining was scored on a scale of 0 to 2 (0, no staining; 1, moderate staining; 2, strong staining).

RESULTS

Quantification of Serum LRG-1 Levels

Serum LRG-1 levels were significantly elevated in patients with PDAC (7.99 [5.07] μg/mL) compared with HVs (3.51 [1.42] μg/mL) and patients with CP (4.96 [2.11] μg/mL) ($P < 0.001$; Fig. 1, Table 1).

When 71 patients with pancreatic cancer were investigated in terms of clinical stage, the mean (SD) LRG-1 concentrations significantly increased with the progression of clinical stage as follows: 3.33 (0.66) μg/mL in stage I, 7.07 (4.18) μg/mL in stage II, 8.74 (5.86) μg/mL in stage III, and 8.72 (4.35) μg/mL in stage IV (Fig. 2). Among them, significant difference of the LRG-1 concentrations was observed between stage I and the other stages, but the differences between stage II and III, III and IV, and II and IV were not significant. The mean (SD) LRG-1 concentration was

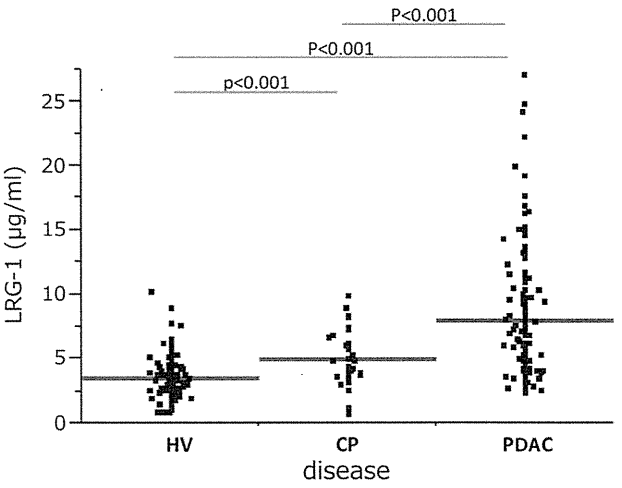

FIGURE 1. Detection of serum LRG-1 by enzyme-linked immunosorbent assay. Serum LRG-1 concentrations were determined for 124 patients with PDAC, 35 patients with CP, and 144 HVs. The mean (SD) LRG-1 concentration for PDAC patient sera was 7.99 (5.07) μg/mL compared with 3.51 (1.42) μg/mL for HV sera ($P < 0.001$) and 4.96 (2.11) μg/mL for CP patients ($P < 0.001$).

TABLE 1. Clinicopathological Characteristics of Patients With PDAC, HVs, and Patients With CP

	PDAC (n = 124)	HV (n = 144)	CP (n = 35)	PDAC vs HV	PDAC vs CP
Age, y*	66.4 (7.83)	44.9 (12.5)	66.7 (10.6)	$P < 0.01$	NS
Sex, % male	70.4	59.3	60.0	NS	NS
LRG-1, μg/mL*	7.99 (5.07)	3.51 (1.42)	4.96 (2.11)	$P < 0.01$	$P < 0.01$
CA19-9, U/mL*	213.7 (208.1)	13.3 (9.61)	7.88 (8.88)	$P < 0.01$	$P < 0.01$
Tumor location (head/body, tail)	42/29				
Lymph node metastasis (positive/negative)	31/40				
Clinical stage (1/2/3/4)†	6/28/21/16				

*Data are presented as mean (SD).

†General Rules for the Study of Pancreatic Cancer, 6th edition (Japan Pancreas Society).[19]

NS indicates not significant.

significantly higher in the lymph node metastasis–positive group than the lymph node metastasis–negative group (9.23 [4.68] μg/mL and 6.51 [4.69] μg/mL, respectively; $P < 0.001$). These results clearly demonstrate that in patients with PDAC, LRG-1 can be associated with local progression or lymph node metastasis of the primary tumor, but not with distant progression, such as liver metastasis. In addition, serum LRG-1 levels positively correlated with CA19-9 ($R^2 = 0.088$, $P < 0.001$), but not with carcinoembryonic antigen ($R^2 = 0.017$, $P = 0.27$).

ROC Curve Analysis and Diagnostic Performance

The ROC curve analysis was performed to determine the clinical usefulness of LRG-1 for diagnosing pancreatic cancer. As shown in Figure 3A, CA19-9 was useful for differentiating patients with PDAC from HVs with AUC of 0.869. At a cutoff value of 37 U/mL for CA19-9, the optimal sensitivity and specificity were 71.9% and 96.4%, respectively. Similar analysis indicated that the AUC for LRG-1 was 0.850. At a cutoff value of 4.81 μg/mL for LRG-1, the optimal sensitivity and specificity were 67.7% and 88.2%, respectively. The combination of LRG-1 and CA19-9 was analyzed by fixing the cutoff value at that of CA19-9. The combination of LRG-1 and CA19-9 improved the differential diagnosis between patients with PDAC and HVs, with an increase in AUC of 0.881 and 75.6% sensitivity and 91.7% specificity.

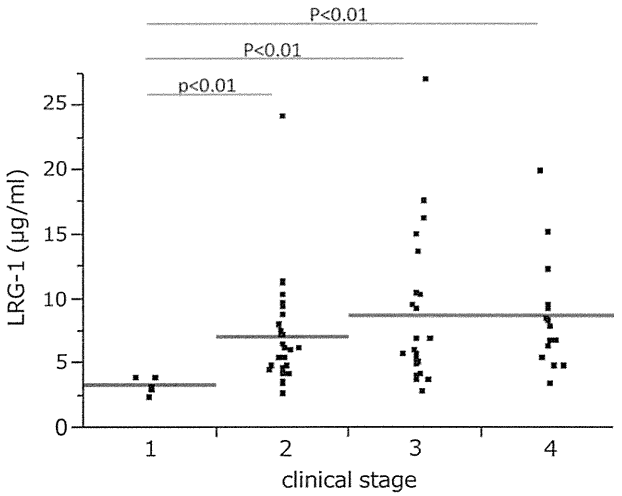

FIGURE 2. Detection of serum LRG-1 in pancreatic cancer patients by enzyme-linked immunosorbent assay. The mean (SD) LRG-1 concentration increased with the progression of clinical stage as follows: stage I, 3.33 (0.66) μg/mL; stage II, 7.07 (4.18) μg/mL; stage III, 8.74 (5.86) μg/mL; and stage IV, 8.72 (4.35) μg/mL. A significant difference was observed between stage I and the other stages, but the differences between stage II and III, III and IV, and II and IV were not significant.

The ability of LRG-1 to make a differential diagnosis between patients with PDAC and patients with CP was also examined. The ROC curve analysis showed that AUC for CA19-9 was 0.913 (Fig. 3B). At a cutoff value of 37 U/mL for CA19-9, the optimal sensitivity and specificity were 73.7% and 100.0%, respectively. Similar analysis for LRG-1 showed AUC of 0.686. At a cutoff value of 4.81 μg/mL for LRG-1, the optimal sensitivity and specificity were 67.7% and 54.3%, respectively. The combination of LRG-1 and CA19-9 enhanced the differentiating power between patients with PDAC and patients with CP, with an increase in AUC to 0.942 with 75.6% sensitivity and 100.0% specificity.

In CA19-9–negative cases, the AUC for LRG-1 was 0.785. At a cutoff value of 4.81 μg/mL for LRG-1, the sensitivity and specificity in differentiating patients with PDAC from HVs were 68.8% and 86.3%, respectively. In regards to differentiating patients with PDAC from patients with CP, the AUC was 0.65, and the sensitivity and specificity were 68.8% and 50.0%, respectively.

LRG-1 Immunohistochemistry

Serum cancer biomarkers can be produced by both cancer cells and the tissues surrounding them. Because LRG-1 is produced in the liver and white blood cells, it is important to identify which cells produce LRG-1 in pancreatic cancer tissue. To address this issue, the pattern of LRG-1 protein expression was assessed by immunohistochemistry using morphological cancer lesions and noncancerous lesions from resection tissue. As shown in Figure 4, pancreatic cancer cells stained positively on the cell surface with a plasma membrane staining pattern, but pancreatic duct cells from noncancer cases, including CP and IPMN, did not stain. However, in CP cases, inflammatory cells such as lymphocytes exhibited weak staining. Positive staining was observed in all cases of pancreatic cancer, but a positive signal was scarcely detected in tissue from CP patients or normal surrounding tissue. Serum LRG-1 levels did not correlate with the intensity of immunostaining ($P = 0.327$).

DISCUSSION

We demonstrated that preoperative serum LRG-1 levels are elevated in patients with PDAC compared with HVs and patients with CP. The LRG-1 level is associated with disease progression and

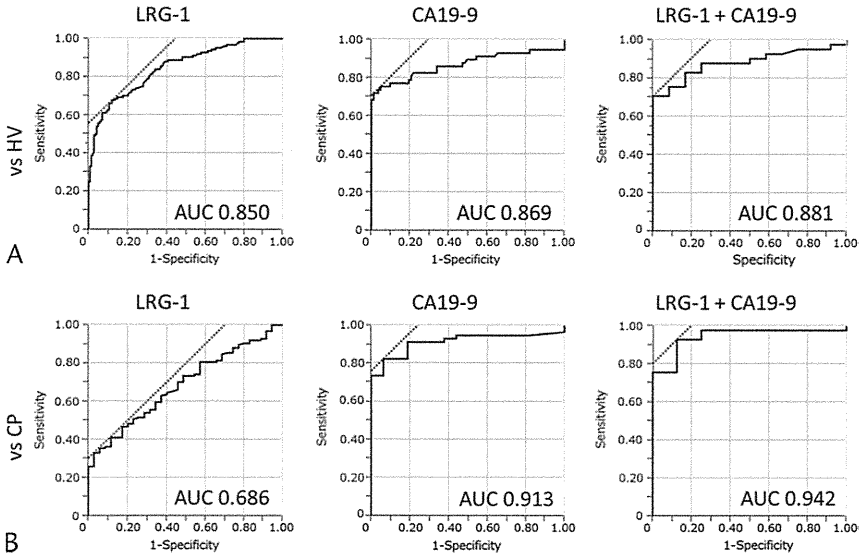

FIGURE 3. Receiver operating characteristic curve analysis for PDAC patients versus HVs (A) and PDAC patients versus CP patients (B). A, Power of LRG-1, CA19-9, and the combination of LRG-1 and CA19-9 in differentiating PDAC patients from HVs. The AUC was 0.850 for LRG-1 alone, 0.869 for CA19-9 alone, and 0.881 for LRG-1 and CA19-9 together. B, Power of LRG-1, CA19-9, and the combination of LRG-1 and CA19-9 in differentiating PDAC patients from CP patients. The AUC was 0.686 for LRG-1 alone, 0.913 for CA19-9 alone, and 0.942 for LRG-1 and CA19-9 together.

lymph node metastasis. Although previous studies have suggested that LRG-1 is induced by both tumor cells and acute/chronic inflammation, serum LRG-1 levels were not as high in the CP patients in our study as previously reported, dues in part to the mild/moderate levels of CP we investigated. Thus, the diagnosis of CP with mild inflammation may be difficult. In contrast, immunohistochemical staining clearly revealed that pancreatic cancer cells, but not noncancer lesions from CP and IPMN cases, were positive for LRG-1. This result suggests that high levels of LRG-1 in pancreatic cancer may be derived from cancer cells or a change in the microenvironment, such as local inflammation associated with cancer progression. However, serum LRG-1 levels did not correlate with staining intensity in tissues, suggesting that serum LRG-1 levels may be dependent on the cleavage of LRG-1 on the cell surface. Andersen et al[9] reported an association between LRG-1 and ovarian cancer. The mean serum LRG-1 concentration was higher in ovarian cancer patients than in healthy women, and highest among stage III/IV patients. Ovarian cancer cells secrete LRG-1, which may contribute directly to the elevated levels of LRG-1 observed in the serum of ovarian cancer patients. Interestingly, the time course is quite different for plasma LRG-1 and carbohydrate antigen 125 (CA125). Suboptimal debulking surgery results in a substantial decrease in CA125, whereas LRG-1 levels remain elevated. Thus serum LRG-1 levels seem to be more directly related to tumor burden than CA125.

Initially, LRG-1 was classified as a marker of acute phase inflammatory responses. Serada et al[15] showed that serum LRG-1 concentrations correlate better with disease activity in ulcerative colitis than C-reactive protein. Kawakami et al[20] reported that LRG-1 levels increase after radiofrequency ablation for hepatocellular carcinoma, suggesting a possible role in the acute stress reaction. We confirmed an association between LRG-1 and C-reactive protein in patients with pancreatic cancer ($R^2 = 0.774$, $P < 0.001$). These dual functions of LRG-1 as a tumor marker and acute phase protein prompted us to use it as a marker of tumor recurrence after an acute phase.

We conducted this study to confirm that LRG-1 can be used as a diagnostic marker for PDAC. We found that LRG-1 can distinguish between patients with PDAC and HVs or patients with CP, but we were not able to demonstrate its effectiveness as an early diagnostic marker. However, we confirmed that serum LRG-1 levels are associated with disease progression and lymph node metastasis, and that pancreatic cancer cells express LRG-1 on the cell surface. An analysis of LRG-1 glycosylation may be a promising approach to distinguish cancer-associated LRG-1 from inflammation-associated LRG-1. In the future, if glycan changes in LRG-1 can be used to identify the pancreatic cancer-specific LRG-1, which is different from the LRG-1 protein made by normal cells such as hepatocytes, it may be a more specific biomarker in PDAC.

Leucine-rich α2-glycoprotein-1 was purified more than 30 years ago. The protein contains 8 typical 24-residue leucine-rich repeats with the consensus sequence.[21] However, the physiological function of LRG-1 is still largely unknown. Shirai et al[22] showed that autologous cytochrome *c* (cyt *c*) is an endogenous ligand of LRG-1 and functions in the detoxification of neurotoxins from snake venom. The expression of LRG-1 is up-regulated during neutrophilic granulocyte differentiation in response to granulocyte-colony stimulating factor, suggesting that LRG-1 is involved in granulopoiesis. Leucine-rich α2-glycoprotein-1 functions as one of the pattern recognition receptors of polymorphonuclear neutrophils and modulates neutrophil function through innate immunity. Serum LRG-1 concentrations correlate well with disease activity in ulcerative colitis. Recent reports have also demonstrated the diagnostic value of LRG-1 in the urine of children with acute appendicitis. These results support the role of LRG-1 in inflammatory responses. Leucine-rich α2-glycoprotein-1 and apoptotic protease activating factor 1 (Apaf-1) have some amino acid sequence homology, and LRG-1 binding to cyt *c* is similar to that of Apaf-1.[23] However, in contrast to Apaf-1, LRG-1 may clear potentially dangerous cyt *c*. This report suggests a role of LRG-1 in preventing lymphocyte death. When bound to extracellular cyt *c* released from apoptotic cells, serum LRG-1 acts as a survival factor for lymphocytes, and possibly other cells. Moreover, aberrant neovascularization contributes to diseases, such as cancer, and is the consequence of inappropriate angiogenic signaling. The epithelial growth factor receptor and vascular

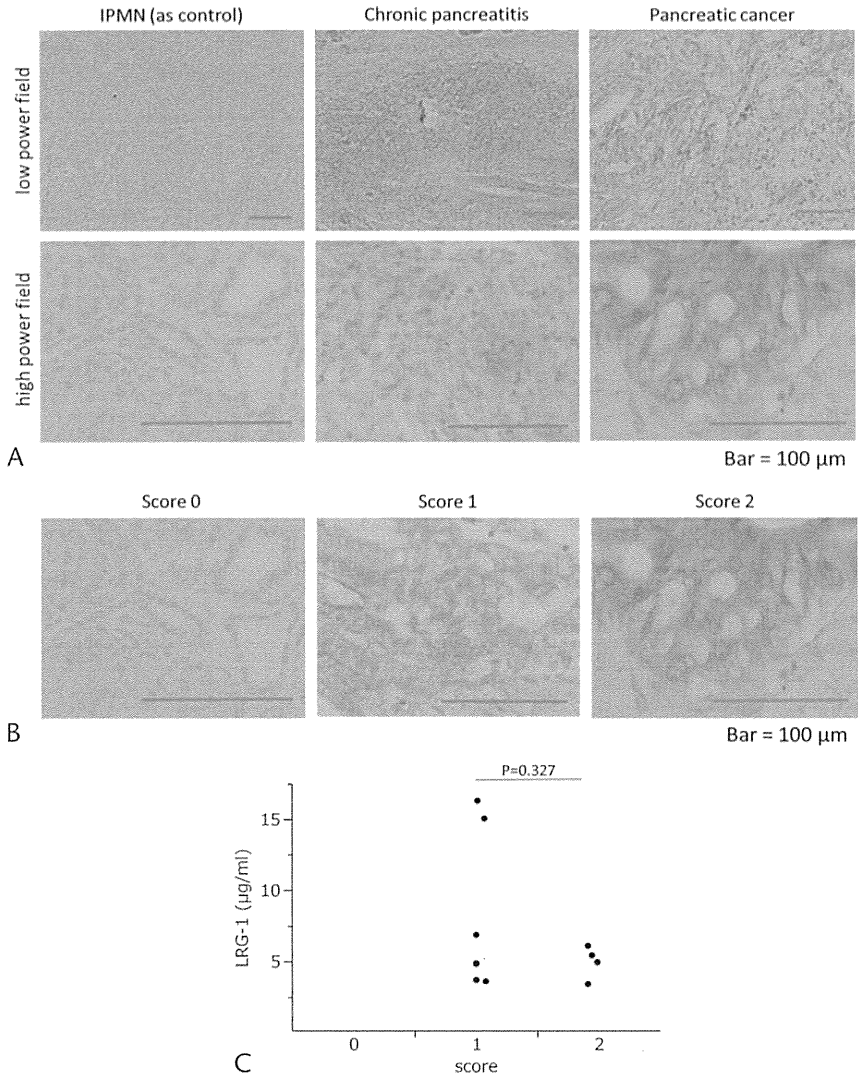

FIGURE 4. Immunohistochemistry of LRG-1 to assess the expression. A, In IPMN cases, the pancreatic duct cells were not stained. In the CP case, the pancreatic duct cells were not stained, but inflammatory cells, such as lymphocytes, stained lightly. In all PDAC cases, the pancreatic cancer cells were stained. Scale bar = 100 μm. B, The intensity of LRG-1 was scored on a scale from 0 to 2 (0, no or faint staining; 1, moderate staining; and 2, strong staining). Scale bar = 100 μm. C, No correlation was found between serum LRG-1 levels and staining intensity ($P = 0.327$).

endothelial growth factor are related to angiogenesis, and targeted agents that are capable of inhibiting signal transduction through epithelial growth factor receptor and vascular endothelial growth factor have already been developed and are used clinically.[24] More recently, LRG-1 was reported to promote angiogenesis by modulating endothelial transforming growth factor β signaling.[25] These results imply that LRG-1 may be a therapeutic target for controlling pathogenic angiogenesis in cancer.

In conclusion, the present study clearly shows that LRG-1 has promise as a novel tumor biomarker for PDAC.

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Serum fucosylated haptoglobin in chronic liver diseases as a potential biomarker of hepatocellular carcinoma development

Abstract

Background: Fucosylation is one of the most important glycosylation events involved in cancer and inflammation. We previously developed a lectin antibody ELISA kit to measure fucosylated haptoglobin (Fuc-Hpt), which we identified as a novel cancer biomarker. In this study, we investigated Fuc-Hpt as a biomarker in chronic liver diseases, especially in hepatocellular carcinoma (HCC).

Methods: We measured serum Fuc-Hpt levels using our ELISA kit in 318 patients with chronic liver diseases, including 145 chronic hepatitis (CH) patients, 81 liver cirrhosis (LC) patients, and 92 HCC patients. During a long-term follow-up period of 7 years (1996–2003), Fuc-Hpt levels were measured at three different time points in 19 HCC patients. Serum Fuc-Hpt levels were also examined with a short-term follow-up period of 3 years (2009–2012) in 13 HCC patients.

Results: Fuc-Hpt levels increased with liver disease progression. Patients with LC and HCC showed significantly increased Fuc-Hpt levels in comparison to CH patients or healthy volunteers. Fuc-Hpt levels tended to be higher in HCC patients than in LC patients. Fuc-Hpt was better than α-fetoprotein (AFP) and AFP-L3 for predicting HCC [diagnosed by computed tomography (CT) or ultrasound] in LC patients with long-term follow-up. More than 80% of LC patients with long-term follow-up showed increased Fuc-Hpt during hepatocarcinogenesis, and 38% of early-stage HCC patients with short-term follow-up showed a gradual increase in Fuc-Hpt before imaging diagnosis.

Conclusions: These results suggest that Fuc-Hpt is a novel and potentially useful biomarker for predicting liver disease progression and HCC development.

Keywords: α-fetoprotein (AFP); fucosylation; lectin antibody ELISA kit; protein induced by vitamin K antagonist II (PIVKA-II).

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Introduction

Oligosaccharide modification is dramatically altered during tumorigenesis. Cancer-specific glycosylation is a potential biomarker for cancer diagnosis. Fucosylation is one of the most important oligosaccharide modifications involved in cancer and inflammation [1]. Several fucosylated proteins have been reported to be potential cancer biomarkers [2–4]. In particular, fucosylated α-fetoprotein (AFP-L3) has been widely examined as a cancer biomarker for hepatocellular carcinoma (HCC) [5]. AFP-L3 is formed by the attachment of a fucose residue to asparagine 232 in the amino acid sequence of AFP. AFP and AFP-L3 are distinguished by the presence of α1-6 fucosylation in AFP-L3. *Lens culinaris* agglutinin (LCA) or lectin recognizes specific carbohydrates, including α1-6 fucose. Lectin-dependent fractionation of AFP was originally described by Breborowicz [6] and Taketa [7]. Aoyagi et al. evaluated the clinical utility of AFP-L3, particularly as a diagnostic tool for HCC [8]. AFP-L3 has been approved as an HCC-specific biomarker. However, the availability of the AFP-L3 diagnostic assay is slightly different between USA and Japan. Marrero et al. reported that a new cut-off value for AFP yielded higher sensitivity than AFP-L3 or protein induced

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by vitamin K antagonist II (PIVKA-II) [9]. This finding was the result of multiple analyses of HCC serum samples. However, there remains insufficient evidence to measure AFP alone as a diagnostic marker for early HCC.

We reported that fucosylated haptoglobin (Fuc-Hpt) is a novel marker for pancreatic cancer and colorectal cancer [10–12]. Further, we investigated the molecular mechanisms underlying its production in patients with pancreatic cancer, and found that increased interleukin-6 (IL-6) production by pancreatic cancer cells induced the production of Fuc-Hpt in the liver [13]. The majority of Hpt found in sera is produced from the liver. However, the liver expresses quite a low level of Fuc-Hpt. To examine Fuc-Hpt as a clinical cancer biomarker, we previously developed a lectin antibody enzyme-linked immunosorbent assay (ELISA) system [14].

The application of Fuc-Hpt as a biomarker of hepatic diseases is complicated by changes in Hpt production due to alteration of liver function. For example, liver cirrhosis (LC) leads to the reduced production of Hpt [15], whereas hepatic inflammation due to chronic hepatitis (CH) causes a dramatic increase in Hpt production. To overcome this issue, we recently reevaluated the sensitivity of our lectin antibody ELISA kit. We found that Fuc-Hpt could be detected in sera that were diluted 25–625 times [16]. In the present study, we examined the clinical utility of serum Fuc-Hpt as a biomarker for HCC. We investigated serum samples from patients with chronic liver diseases and serum samples from patients with HCC before and after diagnosis and therapy. Conventional cancer biomarkers, such as AFP, AFP-L3, and PIVKA-II, were compared with Fuc-Hpt.

Materials and methods

Human subjects

Ninety-two patients with HCC, 81 patients with LC, 145 patients with CH caused by hepatitis B or C infection, and 242 normal volunteers were enrolled. These patients were treated at Osaka National Hospital, Osaka Rosai Hospital, or Osaka University Hospital from 1996 to 2010. A normal volunteer was characterized by the presence of normal liver biochemistry and no history of liver disease or alcohol abuse. LC was diagnosed based on liver histology or clinical, laboratory, and imaging data. The diagnosis of HCC was made either by histopathology or by a combination of imaging tests [ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), or angiography]. Liver biopsy was obtained to confirm the diagnosis in some cases. Nineteen patients with HCC whose sera were collected at three time points during 1996–2003 were defined as long-term follow-up LC–HCC patients. Most of the long-term follow-up patients were treated with transcatheter arterial embolization and/or percutaneous ethanol injection therapy. Thirteen patients with HCC whose sera were collected several times from 2009 to 2012 were defined as short-term follow-up LC–HCC patients. All of the short-term follow-up patients had stage I HCC with tumors that were <2 cm in size and were treated with surgery. Patients with LC were divided into two groups, which included 40 cases that did not develop HCC for at least 5 years (the non-HCC group) and 41 cases that developed HCC (the HCC group). A subset of liver biopsies from patients with CH was analyzed for hepatitis activity. Fuc-Hpt was previously examined in healthy volunteers [16]. In follow-up studies (long- and short-term follow-up), to investigate the usefulness of Fuc-Hpt as a HCC biomarker, the Fuc-Hpt values before and after the diagnosis of HCC were used in the analyses. Sera were maintained at −80 °C. The protocol and informed consent were approved by Institutional Review Boards at Osaka University Hospital, and the study was conducted in accordance with the Helsinki Declaration.

Lectin antibody ELISA for Fuc-Hpt

The Fab fragment of anti-human Hpt IgG (Dako, Carpinteria, CA, USA) was coated onto the bottom of a 96-well ELISA plate, because IgG has the fucosylated oligosaccharide in its Fc portion. The antibody-coated plate was blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin for 1 h followed by washing in PBS containing 0.1% Tween 20 (PBS-T). A 50-μL aliquot of sera, which had been diluted by a factor of 125, was added to each well and incubated for 1 h at room temperature. To detect Fuc-Hpt, 1/1000 diluted biotinylated AAL (*Aleuria aurantia*) was added into each well. As is well known, AAL recognizes both core and outer-arm fucosylation. Therefore, our lectin antibody ELISA system measures both core and outer-arm Fuc-Hpt. The data are presented as ratios relative to the Fuc-Hpt produced by PK8 cells transfected with an expression vector of the Hpt gene, as described previously [13]. Hemoglobin inhibited this lectin antibody ELISA as we reported previously [16]; we therefore excluded hemolysis samples in this study. Limit of the detection is 1.09–70 U/mL in this ELISA kit. Detailed procedures were previously described [13, 14, 16]. The Fuc-Hpt elevation rate was defined as a change in Fuc-Hpt values at two time points during a 10-year follow-up period.

Determination of total haptoglobin, AFP, AFP-L3, and PIVKA-II

Total Hpt levels were measured using a Haptoglobin ELISA kit (ASSAYPRO, St. Charles, MO, USA). A 25-μL aliquot of serum from each patient enrolled in the study was examined according to the manufacturer's protocol. Serum levels of AFP and PIVKA-II were determined using a chemiluminescent enzyme immunoassay (CLEIA). We used the on-chip immunoassay to measure AFP-L3 levels using the micro Total Analysis System of Wako Diagnostics (μ-TAS WAKO) according to the manufacturer's protocol [17].

Statistical analyses

Statistical analysis was conducted using JMP Pro 10.0 software (SAS Institute Inc., Cary, NC, USA). Kruskal-Wallis and Wilcoxon tests were

used to assess any significant differences in continuous clinical or serological characteristics between groups. Differences between groups in the Kaplan-Meier analysis were tested with Wilcoxon's test. A receiver operating characteristic (ROC) curve was generated by plotting sensitivity versus 1 – specificity for every possible cut-off score, and area under the ROC curve (AUC) was calculated. p-Values <0.05 were considered significant.

Results

Serum levels of Fuc-Hpt in patients with chronic liver diseases

Serum Fuc-Hpt levels measured using lectin antibody ELISA were 1314±1574 U/mL in patients with CH, 2433±3262 U/mL in patients with LC, and 3260±3511 U/mL in patients with HCC (Figure 1A). Serum levels of Fuc-Hpt in normal volunteers were 363±482 U/mL. Levels of Fuc-Hpt increased with disease progression. Fuc-Hpt levels were significantly higher in patients with CH and LC compared to those in normal volunteers. Further, Fuc-Hpt levels in LC patients were significantly higher than those in CH patients ($p<0.005$). However, there were no significant differences in Fuc-Hpt levels between patients with LC and HCC ($p=0.061$), although the median level of Fuc-Hpt was slightly higher in HCC patients. ROC analysis indicated that the diagnostic performance of Fuc-Hpt as the HCC biomarker was generally good (HV vs. HCC; AUC 0.84, sensitivity 75.0%, specificity 79.0%, HV, CH and LC vs. HCC; AUC 0.76, sensitivity 40.4%, specificity 76.2%) (Supplementary Data, Figure 1, that accompanies the article at http://www.degruyter.com/view/j/cclm.2015.53.issue-1/issue-files/cclm.2015.53.issue-1.xml). Both graphs indicated that the AUC, sensitivity, and specificity were lower in HCC compared with those observed in PC patients reported in our previous study [16]. Collectively, Fuc-Hpt seems to be a unique biomarker that can help discriminate pathological changes in liver diseases.

Next, we investigated whether Fuc-Hpt levels are associated with the development of HCC from LC. LC patients were divided into two groups. The non-HCC group included LC patients who did not develop HCC during a 5-year follow-up period. The HCC group included all the remaining LC patients. Fuc-Hpt levels were lower in patients in the non-HCC group than those of patients in the HCC group, although the differences were not statistically significant (Figure 1B). A few patients in the HCC group had extremely high Fuc-Hpt levels, none of the patients in the non-HCC group had the high Fuc-Hpt levels that were observed in these few outlying cases in the HCC group.

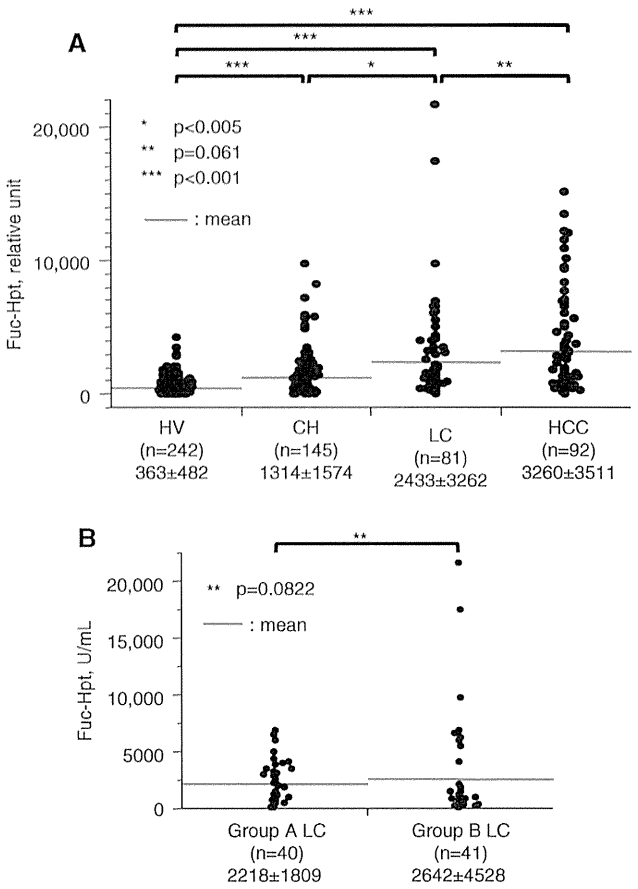

Figure 1 Levels of serum Fuc-Hpt in patients with chronic liver diseases.
(A) Fuc-Hpt levels were measured with a lectin antibody ELISA kit. Values are shown in relative units. (B) Patients with LC were divided into two groups. LC patients in the non-HCC group did not develop HCC within a 5-year period. The HCC group included all other LC patients. In each panel, the gray bars indicate mean values, and n indicates the numbers of patients in each group.

Comparison of serum Fuc-Hpt among patients with LC and CH

Serum Fuc-Hpt levels were investigated in patients with CH who underwent liver biopsy to assess liver histology (n=62). CH patients who underwent liver biopsy were stratified according to hepatitis pathological activity. The activity of CH was determined by pathological analysis according to the METAVIR scoring system and divided into A0 and A1-3 [18]. Fuc-Hpt levels in patients with CH (A0) were significantly lower than those in patients with CH (A1-3) (Figure 2A). In addition, we compared the histological scores and clinical data of our subjects (Supplementary Data, Table 1A, B and Figure 2). We found that serum Fuc-Hpt levels tended to correlate with activity scores, but did not correlate with fibrosis stage scores divided by METAVIR scoring system (F0-4) [18]. Interestingly, the

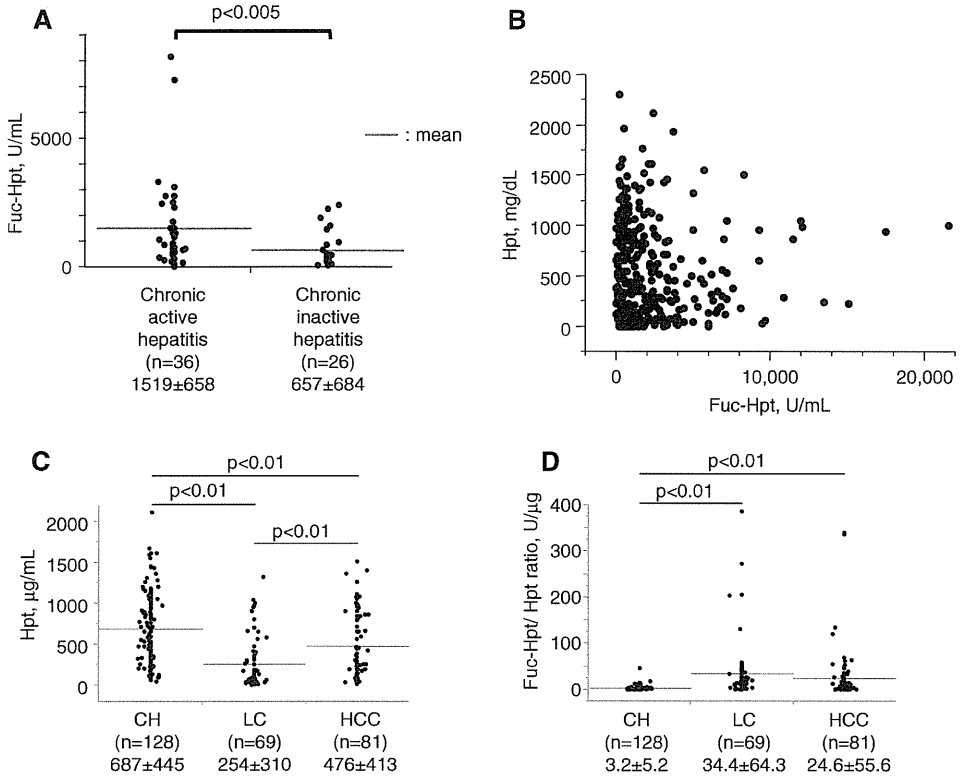

Figure 2 Comparison of serum Fuc-Hpt levels in CH and LC.
(A) Serum Fuc-Hpt levels in patients with chronic active and inactive hepatitis. (B) Fuc-Hpt and total Hpt levels were compared in 318 patients with chronic liver diseases. (C) Serum haptoglobin (Hpt) levels were measured using an ELISA kit in chronic liver disease patients. (D) Fuc-Hpt and Hpt ratio in chronic liver disease patients. In each panel, the gray bars indicate mean values, and n indicates the numbers of patients in each group.

ratio of Fuc-Hpt to total Hpt correlated significantly with activity scores. As Hpt is an acute-phase protein produced in the liver, we compared Fuc-Hpt and total Hpt levels. However, there was no correlation between total Hpt and Fuc-Hpt levels (Figure 2B), suggesting that secretion mechanisms of Hpt and Fuc-Hpt were different, and measuring the Fuc-Hpt level could be valuable instead of measuring Hpt level alone. Next, we compared serum Hpt levels in chronic liver disease patients (Figure 2C). We found that serum Hpt levels were significantly decreased in LC patients compared to levels in CH patients as previously reported [15]. Hpt levels were significantly higher in HCC patients than in LC patients. We also investigated the relationships between serum Fuc-Hpt levels and several variables (alanine aminotransferase, albumin, total bilirubin, prothrombin time [%]) in limited numbers of patients from whom we could obtain clinical data (Supplementary Data, Figure 3). We found no significant relationships between serum Fuc-Hpt levels and several variables in both LC and HCC patients. The Fuc-Hpt/Hpt ratio was significantly increased in LC and HCC patients compared to that in CH patients (Figure 2D).

Fuc-Hpt in LC–HCC follow-up patients

Serum Fuc-Hpt levels were examined at three time points during a 10-year period in 19 patients who progressed from LC to HCC (Table 1, Supplementary Data, Figure 4). Interestingly, 80% of cases showed increased Fuc-Hpt levels after HCC was detected. However, we were unable to determine the cut-off value for the development of HCC. More than 70% of patients showed a 3- to 5-fold increase in Fuc-Hpt. We evaluated Fuc-Hpt as a diagnostic marker for HCC compared to other conventional cancer biomarkers for HCC, such as AFP, AFP-L3, and PIVKA-II. The AUC values for HCC prediction by Fuc-Hpt, PIVKA-II, AFP, and AFP-L3 were 0.840, 0.696, 0.602, and 0.530 (%), respectively (Table 2). These results indicated that the diagnostic ability of Fuc-Hpt was superior to that of other classical HCC diagnostic markers. The cut-off values for each marker were determined by ROC analysis. Combined analysis of Fuc-Hpt and other tumor markers did not increase the AUC value for HCC diagnosis. Next, we investigated the Fuc-Hpt elevation rate in these patients to determine if increased Fuc-Hpt is predictive for HCC. The

Table 1 Serum Fuc-Hpt levels changes in long-term follow-up patients.

	Patient 1			Patient 2			Patient 3			Patient 4			Patient 5		
Liver disease status	**CH**	**LC**	**HCC**	**CH**	**LC**	**HCC**	**LC**	**HCC**	**HCC**	**CH**	**LC**	**HCC**	**LC**	**HCC**	**No HCC**
Time spans, years	0	3	5.3	0	4	6	0	3.8	6.4	0	4.5	6	0	3.3	5.6
Fuc-Hpt, U/mL	197	4123	4010	1172	1584	2350	99	438	583	0	931	2469	548	2730	350
Hpt, μg/mL	530	0	13	1060	1373	694	0	0	39	0	13	242	2	877	0
AST, U/L	89	45	50	44	63	91	56	45	36	89	89	103	47	21	50
ALT, U/L	79	36	35	42	86	117	59	40	26	84	88	94	38	15	47
GGT, U/L	73	103	60	137	110	100	63	40	32	63	70	75	56	33	108
Alb, g/dL	N.A.	3.5	N.A.	N.A.	4	3.8	3.5	3.4	2.6	4	3.6	3.9	3.1	3.2	3.5
CHE, IU/L	55	N.A.	50	109	120	104	69	75	48	134	135	136	108	77	167
Plt, $\times 10^{10}$/L	8.6	9.4	8.6	16.1	17.1	15.5	6.8	5.1	7.3	12.9	12.7	13.2	8.5	20.8	9.7
T-Bil, mg/dL	0.7	1.2	1.1	1.6	0.8	0.9	1.3	1.3	1.3	0.8	0.6	0.7	N.A.	1	1.2
D-Bil, mg/dL	0.7	1.2	1.1	N.A.	N.A.	N.A.	0.9	0.3	0.6	0.2	N.A.	N.A.	N.A.	0.2	0.4
AFP, ng/mL	N.A.	N.A.	91	8	N.A.	31.4	6	N.A.	4	4	6	7.4	N.A.	4	N.A.
PIVKA-II, AU/mL	N.A.	N.A.	N.A.	N.A.	33	N.A.	1.33	N.A.	N.A.	N.A.	22	21	<10	N.A.	38

AFP, alpha fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHE, choline esterase; D-Bil, direct bilirubin; Fuc-Hpt, fucosylated haptoglobin; GGT, gamma glutamyl transpeptidase; Hpt, haptoglobin; T-Bil, total bilirubin.

Table 2 Availability of several types of cancer biomarkers for diagnosis of HCC in long-term follow-up patients.

	Fuc-Hpt, U/mL	PIVKA-II, AU/mL	AFP, ng/mL	AFP-L3, %	Fuc-Hpt+ PIVKA-II	Fuc-Hpt+ AFP	Fuc-Hpt+ AFP-L3, %
AUC	0.840	0.696	0.602	0.530	0.840	0.836	0.841
Sensitivity, %	68.8	56.3	85.7	66.7	62.5	81.3	66.7
Specificity, %	87.5	87.5	50.0	54.5	43.8	43.8	42.4
PPV, %	84.6	81.8	63.2	61.5	90.9	76.5	88.9
NPV, %	73.7	66.7	77.8	60.0	71.4	80.0	71.4
Cut-off value	1655.4	69.0	13.0	5.0	–	–	–

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

Fuc-Hpt elevation rate, which was defined as a change in Fuc-Hpt values at two time points during a 10-year follow-up period, was significantly higher in HCC(+) patients with LC than in HCC(−) patients with LC (Figure 3A). ROC analysis indicated that a Fuc-Hpt elevation rate of more than 498.2% showed 100% specificity for HCC detection (Figure 3B). We found in this study that the Fuc-Hpt elevation rate was significant for the prediction of HCC occurrence in each patient.

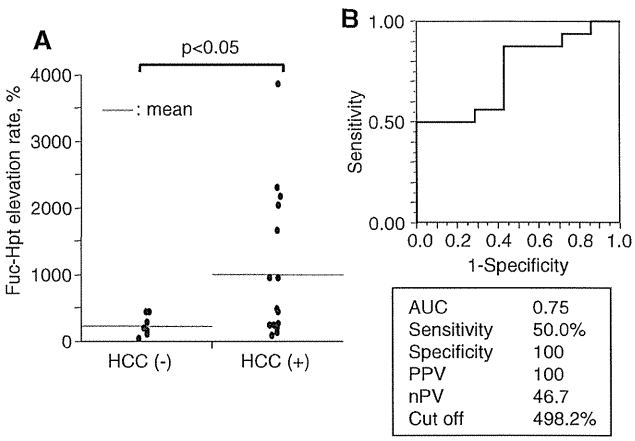

Figure 3 Serum Fuc-Hpt levels were compared with LC alone and LC plus HCC in LC–HCC follow-up patients.
(A) Fuc-Hpt elevation rates were measured in 19 patients with HCC before and after HCC development. Gray bars indicate mean values.
(B) ROC analysis of data in panel A. AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.

Which cancer biomarkers are useful for early diagnosis of HCC?

Fuc-Hpt levels were investigated in 13 patients with early-stage HCC who underwent surgery. Fuc-Hpt was measured every 6 months before and after HCC detection for 2–3 years. Results are summarized in Table 3. The AUC values

Table 3 Availability of several types of cancer biomarkers for diagnosis of HCC in short-term LC–early HCC follow-up patients.

	Fuc- Hpt, U/mL	PIVKA –II, AU/mL	AFP, ng/mL	AFP-L3, %	Fuc-Hpt+ PIVKA-II	Fuc-Hpt+ AFP	Fuc-Hpt+ AFP-L3, %
AUC	0.618	0.658	0.546	0.555	0.642	0.672	0.636
Sensitivity, %	50.0	66.7	33.3	41.7	66.7	83.3	75.0
Specificity, %	76.3	73.3	93.6	73.7	63.3	54.8	57.9
PPV, %	40	50	66.7	33.3	42.1	41.7	36.0
NPV, %	82.9	84.6	78.4	80	82.6	89.5	88.0
Cut-off value	375	28.0	14.0	5.2	–	–	–

of Fuc-Hpt, PIVKA-II, AFP, and AFP-L3 were 0.618, 0.658, 0.546, and 0.555, respectively. Although AUC values of Fuc-Hpt and PIVKA-II were higher than those of AFP and AFP-L3, none of the cancer biomarkers was sufficient to make an early diagnosis of HCC. Combination analysis of Fuc-Hpt and PIVKA-II, AFP, or AFP-L3 was also not effective for HCC prediction. In contrast, approximately 40% of HCC patients showed gradual increases in Fuc-Hpt and PIVKA-II. Representative cases that showed the utility of cancer biomarkers for detecting early HCC are summarized in Supplemental Data. Among 13 patients with HCC, cases 1–5 showed gradual increases in Fuc-Hpt before HCC was detected by ultrasound or CT. Peak increases in Fuc-Hpt were observed 1–1.5 years before HCC detection in cases 6–10 followed by decreased Fuc-Hpt at the time of HCC diagnosis. Fuc-Hpt did not show utility as a cancer biomarker in cases 11–13. PIVKA-II was useful as a biomarker for early HCC in five cases (1, 4, 6, 7, and 11). AFP was useful as a biomarker for early HCC in cases 5, 9, and 13. In contrast, AFP-L3 was useful for early HCC detection only in case 13. Although the specificity of HCC prediction by AFP was very high, the sensitivity was only 33.3%. In addition, gradual increases in AFP were observed in 3/13 cases (23%). Quantitative analyses of AFP-L3 and AFP-L3% (data not shown) were not effective for early HCC prediction. Although HCC patient numbers investigated in the present study were limited, our study demonstrated that Fuc-Hpt is a more useful biomarker for early diagnosis of HCC than other conventional biomarkers.

Discussion

In the present study, we have demonstrated that Fuc-Hpt is a novel and unique biomarker that can discriminate between pathological changes occurring in chronic liver diseases. Serum Fuc-Hpt levels increased with the progression of liver disease, suggesting that Fuc-Hpt levels may be dependent on liver functions and/or fibrosis. Our previous study already demonstrated that hepatoma cell lines (Hep3B and HepG2) produce Fuc-Hpt [13]. In addition, the present retrospective analysis showed increased levels of Fuc-Hpt over time, indicating its potential utility as a predictive biomarker of HCC. Surprisingly, this potential utility of Fuc-Hpt was superior to other classical HCC biomarkers (AFP, AFP-L3, PIVKA-II) (Table 2). In particular, Fuc-Hpt elevation rates were useful for the diagnosis of HCC development, and a Fuc-Hpt elevation rate of more than 498.2% showed 100% specificity for HCC detection (Figure 3). Although none of these HCC biomarkers (Fuc-Hpt, AFP, AFP-L3, PIVKA-II) were sufficiently able to aid in the early diagnosis of HCC in this study, Fuc-Hpt showed gradual increases in five of 13 (38%) patients prior to imaging HCC diagnosis (Table 3, Supplementary Data, Figure 5). In addition, Fuc-Hpt levels were significantly higher in patients with chronic active hepatitis than in those with inactive hepatitis (Figure 1B, Supplementary Data, Table 1A), suggesting that inflammation may be involved in Fuc-Hpt induction. Consistent with this hypothesis, inflammation-related cytokines, such as IL-6, have been shown to induce Fuc-Hpt production [13]. However, total Hpt and Fuc-Hpt levels were not correlated, suggesting that the production of Hpt is not always dependent on serum Fuc-Hpt levels. As we reported previously, secretion of fucosylated proteins in the liver is selectively regulated by cellular polarity [19, 20], which may also affect serum Fuc-Hpt levels in liver diseases.

Indeed, serum Hpt levels are increased with inflammation because IL-6 receptor stimulates the production of Hpt in the liver. However, in cases with LC patients, the production of Hpt in the liver is decreased due to liver dysfunction as described previously [15]. Hpt levels were significantly higher in HCC patients than in LC patients. In this study, the mean value of Hpt in HCC patients was higher than that in LC patients and lower than that in CH patients. Therefore, these results demonstrated that serum Hpt levels are not suitable markers for the prediction of HCC occurrence in CH patients. This study demonstrated that serum Fuc-Hpt levels were also correlated

with liver inflammation in patients who had undergone liver biopsy. The mean value of serum Fuc-Hpt levels increased with the progression of liver disease. We would like to investigate the precise relationships between clinical data (including histological data) and serum Fuc-Hpt levels in future studies.

Routine follow-up of patients with liver diseases includes imaging by ultrasonography or CT every 3–6 months. Patients with LC should be closely followed to monitor HCC development. The present study demonstrates the utility of Fuc-Hpt and conventional cancer biomarkers, such as AFP and PIVKA-II. However, as previously noted, it is difficult to apply biomarkers for the early diagnosis of HCC. Although the AUC values of Fuc-Hpt and PIVKA-II were <0.6, these biomarkers detected HCC before imaging diagnosis in only 30%–40% of patients with early-stage HCC (Supplementary Data, Figure 5). Interestingly, increased levels of AFP showed the highest specificity in short-term follow-up analyses. Gradual increases in AFP are recognized as a sign of HCC development, consistent with the results of this study. Serum Fuc-Hpt levels >1655.4 U/mL and Fuc-Hpt elevation rates >498.2% showed high specificity (87.5% and 100%) for diagnosing HCC in patients with LC. The high specificity suggests that Fuc-Hpt may be produced from HCC cells but not from hepatocytes in the cirrhotic liver. Moreover, fucosylation is a possible signal for the polarized secretion of fucosylated glycoproteins into bile ducts in the liver, as we have previously reported [19]. Therefore, the deformity of hepatocyte polarity in cancer cells would cause increased production or secretion of Fuc-Hpt into the serum in HCC patients.

Fuc-Hpt may be a useful predictive marker for HCC. The non-HCC group of LC patients with low levels of Fuc-Hpt did not develop HCC during a 5-year follow-up. In summary, serum Fuc-Hpt is a biomarker that can discriminate between pathological changes in liver diseases and showed value as a potential predictive biomarker for HCC. Based on these promising data, we have initiated a prospective study with a larger group of patients with LC to validate Fuc-Hpt as a novel diagnostic marker for HCC.

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N-Acetylglucosaminyltransferase V exacerbates concanavalin A-induced hepatitis in mice

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Abstract. *N*-Acetylglucosaminyltransferase V (GnT-V) catalyzes β1-6 branching in asparagine-linked oligosaccharides and is one of the most important glycosyltransferases involved in carcinogenesis, cancer metastasis and immunity. To investigate the biological functions of GnT-V, the present study developed GnT-V transgenic (Tg) mice and the role of GnT-V in experimental immune-mediated hepatitis, induced by concanavalin A (ConA), were investigated. It was found that the aberrant expression of GnT-V exacerbated ConA-induced hepatitis in the Tg mice compared with the wild-type (WT) mice. The survival rate of the ConA-induced hepatitis at a high-dose of ConA was significantly lower in the Tg mice. Intravenously injected ConA is known to initially bind predominantly to the mannose gland of the liver sinusoidal endothelial cell (LSEC) surface and to leads to the activation of various immune cells. In the present study, the binding affinity of ConA to the LSECs did not differ between the WT and Tg mice. In addition, T cell receptor stimulation by anti-cluster of differentiation (CD)3/CD28 antibodies produced lower levels of T helper (Th)1 cytokine (interferon-γ) and higher levels of Th2 cytokine (interleukin-10) in the Tg mouse splenic lymphocytes compared with WT mice. The composition of the hepatic mononuclear cells revealed that CD11b-positive cells were significantly increased in the GnT-V Tg mice. In addition, F4/80-positive cells were significantly increased in the Tg mouse liver and the depletion of macrophages reduced the difference in the severity of ConA-induced hepatitis between the WT and Tg mice. In conclusion, the present findings indicated that the aberrant expression of GnT-V led to an increase in hepatic macrophage infiltration and enhanced ConA-induced hepatitis. Modulation of glycosylation may be a novel therapeutic target for immunity-associated acute hepatitis.

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Abbreviations: CCL2, C-C chemokine ligand 2; ConA, concanavalin A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GnT-V, *N*-acetylglucosaminyltransferase V; IFN-γ, interferon-γ; IL, interleukin; LSEC, liver sinusoidal endothelial cell; NK cell, natural killer cell; TCR, T cell receptor; TNF-α, tumor necrosis factor-α

Key words: glycosylation, *N*-glycan, macrophage, galectin-3, T cell receptor

Introduction

Concanavalin A (ConA) is a lectin that induces hepatitis through the modulation of various immune cells, including macrophages, T cells, natural killer (NK) cells and liver sinusoidal endothelial cells (LSECs) (1-4). Initially, intravenously injected ConA binds predominantly to the mannose gland of the LSEC surface and subsequently leads to the breakdown of the LSECs (5-7). Damaged LSECs produce inflammatory cytokines and chemokines, and LSEC detachment facilitates the binding of ConA to macrophages. T cells recognize the ConA-modified major histocompatibility complex class II and T cell receptor (TCR) of macrophages and are subsequently activated (4,8,9). In addition, these immune cells produce various inflammatory cytokines and chemokines, including interferon-γ (IFN-γ) (10,11), interleukin-2 (IL)-2 (11), IL-4 (12), IL-6 (12), C-C chemokine ligand 2 (CCL2) (13) and CXC-motif chemokine ligand 12 (9). Anti-inflammatory cytokines, including IL-10, are also involved in ConA-induced hepatitis. Among these biologically active substances, IL-10 has been reported to protect the liver from ConA-induced hepatitis (14,15).

Previous findings in glycobiology have provided direct evidence of the involvement of oligosaccharide changes in human diseases (16). Oligosaccharide modification of glycoproteins is predominantly divided into two types: *N*-glycans, attached to the asparagine residues, and *O*-glycans, attached to the serine/threonine residues (17) The branching formation on *N*-glycans is one of the most important factors regulating

the biological functions of oligosaccharides and terminal modifications, including sialylation and fucosylation (17). The branching formation on *N*-glycans is regulated by several types of *N*-acetylglucosaminyltransferases and the upregulation or downregulation of these glycosyltransferases may modify the biological functions of adhesion molecules and the signaling pathways of several growth factor receptors (18).

N-Acetylglucosaminyltransferase V (GnT-V) is involved in the synthesis of β1-6 GlcNAc branching formation on *N*-glycans (19). It is well-established that GnT-V is one of the most important glycosyltransferases involved in cancer metastasis (20), promoting cancer metastasis through the enhancement of growth factor signaling, integrin function and the expression of certain types of proteases (19-21). GnT-V also has important functions in the immune system. Deficiency of GnT-V in mice leads to an autoimmune disease phenotype and GnT-V-induced TCR oligosaccharide modification suppresses TCR signaling (22,23). These findings indicate that GnT-V decreases inflammatory responses through suppression of T cell activation. While the expression of GnT-V is low in the normal liver, it is increased during the progression of chronic disease and liver regeneration (24,25). Our previous study indicated that the expression of GnT-V in the normal mouse liver is higher in hepatic non-parenchymal cells, including immune cells, compared with hepatocytes (26). These findings indicate that GnT-V is important in the progression of liver diseases.

Considering these findings, GnT-V is expected to be important in the progression of liver disease through modulation of the immune system. However, the significance of changes in GnT-V-induced glycosylation in liver diseases remains to be elucidated. To address this issue, the present study investigated the role of GnT-V in experimental immune hepatitis using a mouse ConA hepatitis model.

Materials and methods

Mice. GnT-V (*Mgat5*) transgenic (Tg) mice (β-actin promoter; C57BL/6J background) were produced, as previously described (27). In the present study, wild type (WT) litter-mates were used as control mice. The animals were provided with unrestricted access to food and water, housed in temperature- and humidity-controlled rooms and maintained in a 12/12 h light/dark cycle. All experiments were performed using 8-12 week old male mice. At the end of each experimental period, blood was drawn aseptically from the inferior vena cava and centrifuged at 13,000 x g for 5 min at 4˚C to collect the serum. Mice were anesthetized via intraperitoneal injection of pentobarbital (50 mg/kg), and the livers were subsequently removed and fixed with 10% buffered paraformaldehyde (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), flash frozen in liquid nitrogen for protein and mRNA extraction was performed as described previously (26). All experimental procedures described in the present study were approved by the Ethics Review Committee for Animal Experimentation of the Osaka University School of Medicine (Osaka, Japan).

ConA-induced hepatitis. ConA (Sigma-Aldrich, St. Louis, MO, USA) at 12.5 mg/kg body weight (BW) was dissolved in 200 μl phosphate-buffered saline (PBS; Sigma-Aldrich) and injected into WT and GnT-V Tg mice through the tail vein. Serum alanine aminotransferase (ALT) concentrations were measured using a Transaminase CII-test Wako kit (Wako Pure Chemical Industries, Tokyo, Japan). To examine the survival rate of the rats, a relatively high dose of ConA (20 mg/kg/BW) was injected intravenously.

Hematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase dUTP nick end labeling *(TUNEL) and immunohistochemical staining.* Liver sections were stained with H&E, Apop Tag® Peroxidase *in Situ* Apoptosis Detection kit, according to the manufacturer's instructions (Merck Millipore, Darmstadt, Germany), or monoclonal human anti-F4/80 antibody (1:100; HCA154; Bio-Rad AbD Serotec, Oxford, UK).

Cytokine analysis. Splenic lymphocytes were collected, as previously described (28). The cells ($1x10^6$ cells) were cultured in flat-bottom 96-well culture plates for 48 h in RPMI-1640 (Sigma-Aldrich) with 10% fetal bovine serum (Sigma-Aldrich) and antibiotics/antimycotics in the presence of anti-mouse cluster of differentiation (CD)3 (5 μg/ml) and anti-mouse CD28 antibodies (5 μg/ml; BD Biosciences, San Jose, CA, USA). The culture supernatant was collected and production of the IFN-γ and IL-10 cytokines were determined by enzyme-linked immunosorbent assay (ELISA; eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The levels are expressed as the mean ± standard deviation of $1x10^6$ cells. These cells ($3x10^6$ cells) were also cultured in flat-bottom 96-well culture plates for 24 h in RPMI-1640 with 10% fetal bovine serum and antibiotics/antimycotics in the presence of ConA (5 μg/ml). The culture supernatant was collected and the production of IFN-γ and IL-10 cytokines was determined by ELISA (eBioscience), according to the manufacturer's instructions. The levels are expressed as the mean ± standard deviation of $3x10^6$ cells.

Quantification of gene expression levels. Total RNA was extracted from cells with a QIAshredder and an RNeasy Mini kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany) and transcribed into complementary DNA with a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Reverse transcription quantitative polymerase chain reaction was performed with a Thunderbird SYBR qPCR mix (Toyobo) using specific primers on a LightCycler according to the instructions provided by the manufacturer (Roche Diagnostics, Indianapolis, IN, USA). The cycling conditions were as follows: 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primers used were as follows: *Ifn-γ* (cat. no. QT01038821), *Il-10* (cat. no. QT00106169), *Ccl2* (cat no. QT01747165), *Ccl5* (cat. no. QT00167832) and *18s* rRNA (cat. no. QT01036875; Qiagen). The primers for *T-bet, Gata-3* and *Galectin-3* were purchased from Sigma-Aldrich and the sequences were as follows: *T-bet,* sense 5'-GCC AGG GAA CCG CTT ATA TG-3' and antisense 5'-GAC GAT CAT CTG GGT CAC ATT GT-3'; *Gata-3,* sense 5'-TTA TCA AGC CCA AGC GAA G-3' and antisense 5'-TGG TGG TGG TCT GAC AGT TC-3' and *Galectin-3,* sense 5'-CAG GAT TGT TCT AGA TTT CAG G-3' and antisense 5'-TTG TCC TGC TTC

GTG TTA CAC-3'. The mRNA expression levels were normalized to the mRNA expression level of *18s* and expressed in arbitrary units.

Isolation of mouse LSECs. LSECs were isolated from WT and GnT-V Tg mice by performing a two-step collagenase-pronase perfusion of their livers, as described previously (29). Briefly, the livers were perfused for 3 min at room temperature at a flow rate of 4 ml/min with SC-1 solution containing 8,000 mg/l NaCl, 400 mg/l KCl, 78 mg/l NaH_2PO_4 $2H_2O$, 151 mg/l Na_2HPO_4 $12H_2O$, 2380 mg/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 350 mg/l $NaHCO_3$, 190 mg/l ethylene glycol tetraacetic acid and 900 mg/l glucose (pH 7.2), followed by digestion at 37˚C for 3 min with 0.053% pronase and 0.027% collagenase dissolved in an SC-2 solution containing 8,000 mg/l NaCl, 400 mg/l KCl, 78 mg/l NaH_2PO_4 $2H_2O$, 151 mg/l Na_2HPO_4 $12H_2O$, 2,380 mg/l HEPES, 350 mg/l $NaHCO_3$ and 735 mg/l $CaCl_2$ $2H_2O$ (pH 7.5). Each digested liver was excised and cut into 2-mm sections. The resulting suspension was filtered through a 100 μm cell strainer and centrifuged at 50 x g for 1 min at 4˚C to remove hepatocytes. This protocol was repeated three times and the supernatants were centrifuged at 300 x g for 10 min at 4˚C. The pellet was washed and suspended in Dulbecco's modified Eagle's medium twice. Non-parenchymal cells were further separated from parenchymal cells by density-gradient centrifugation at 1,500 x g for 20 min at 4˚C on a 30% Histodenz cushion (Sigma-Aldrich). The LSECs were then isolated by magnetic cell sorting using magnetic beads (MACS; Miltenyi Biotec, Gladbach, Germany) with rat anti-mouse CD31 antibody (11-0311-85; eBioscience), according to the manufacturer's instructions. The purity of the MACS-enriched LSECs was assessed by flow cytometric analysis with rat anti-mouse CD31 antibody (11-0311-85; eBioscience) using a FACS Canto II (BD Biosciences).

Western and lectin blot analyses. Immunoblotting was performed, as described previously (26). Briefly, ($2x10^6$) isolated mouse LSECs were lysed with 1% Triton X-100 and ~10 mg frozen liver tissue was then lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS). The samples were then subjected to heat denaturation at 98˚C for 5 min, separated with SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then blocked with either 5% skimmed milk for western blotting or 3% bovine serum albumin for lectin blotting. The following antibodies were used for immunodetection: Rabbit polyclonal anti-GnT-V (24D11; 1:3,000; Fujirebio, Tokyo, Japan), leukoagglutinating phytohemagglutinin (L_4-PHA) lectin (1:1,000; cat. no. J112; J-Oil Mills, Inc., Tokyo, Japan), ConA lectin (1:1,000; cat. no. J103; J-Oil Mills, Inc.) and rabbit polyclonal anti-GAPDH (1:3,000; cat. no. 2275-PC-1; Trevigen, Gaithersburg, MD, USA). Immunoreactive bands were visualized on an GE Healthcare film using Amersham Enhanced Chemiluminscence Western Blotting Detection reagents (GE Healthcare, Waukesha, WI, USA).

Isolation of mouse hepatocytes and liver mononuclear cells (MNCs). Mouse hepatocytes and MNCs from the liver were prepared, as previously described (30). Briefly, the mice were anesthetized and their abdomens were opened. The inferior vena cava and portal vein were cut to enable blood outflow. The liver was removed and gently passed through a stainless steel mesh. The liver cell suspension was collected and the hepatocytes were separated from the MNCs by centrifugation at 50 x g for 1 min. This procedure was repeated three times and the supernatants were centrifuged at 150 x g for 7 min at 4˚C. The MNC populations were purified by centrifugation through a Percoll gradient. The cells were collected, washed in PBS and resuspended in 40% Percoll (Sigma-Aldrich). The cell suspension was gently overlaid onto 70% Percoll (Sigma-Aldrich) and centrifuged for 15 min at 1,400 x g. The MNCs were collected from the interface and were washed twice in PBS for use in subsequent analysis.

Flow cytometric analysis of hepatic MNCs and LSECs. ConA (12.5 mg/kg/BW; Sigma-Aldrich) dissolved in 200 μl PBS was injected into the WT and GnT-V Tg mice through the tail vein. After 2 h, the liver MNCs were isolated, as described above. The liver MNCs, stained with monoclonal rat anti-mouse CD4 (4800041-82), rat anti-mouse CD8α (11-0081-82), mouse anti-mouse CD19 (17-0191-829, rat anti-mouse CD11b (12-0112-82), hamster anti-mouse CD11c (48-0114-82), and mouse anti-mouse NK1.1 (17-5941-82) antibodies (eBioscience) and the LSECs, stained with anti-CD31 antibody, fluorescein isothiocyanate-labeled L_4-PHA (J512) and ConA (J503) (J-Oil Mills Inc.) were subjected to flow cytometric analysis using a FACS Canto II™ (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software version 7.6.1 (TreeStar Inc., Ashland, OR, USA). The detailed procedure has been described previously (31).

Depletion of macrophages. The suicidal liposome technique has been used previously to deplete macrophages (32). Clodronate-liposomes were purchased from Clodronate Liposomes, (VUmc FdG, Amsterdam, Netherlands). Briefly, the mice were injected with 200 μl clodronate-liposomes through the tail vein. The injection was performed 2 days prior to the ConA administration.

Statistical analysis. Statistical analysis was performed using JMP Pro 10.0 software (SAS Institute Inc., Cary, NC, USA). Kaplan-Meier curves were used to demonstrate the survival rates. The results are expressed as the mean ± standard deviation. Groups of data were compared by the Wilcoxon test for non-parametric data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ConA-induced hepatic injury is exacerbated in GnT-V Tg mice compared with WT mice in vivo. ConA (12.5 mg/kg/BW) was injected intravenously into WT and GnT-V Tg mice. Histological analyses of the liver tissue sections indicated that the GnT-V Tg mice were more sensitive to ConA-induced hepatic injury (Fig. 1A). Liver tissue sections in the GnT-V Tg mice exhibited more widespread necrotic areas compared with the WT mice. In addition, liver tissue sections from the GnT-V Tg mice exhibited increased numbers of

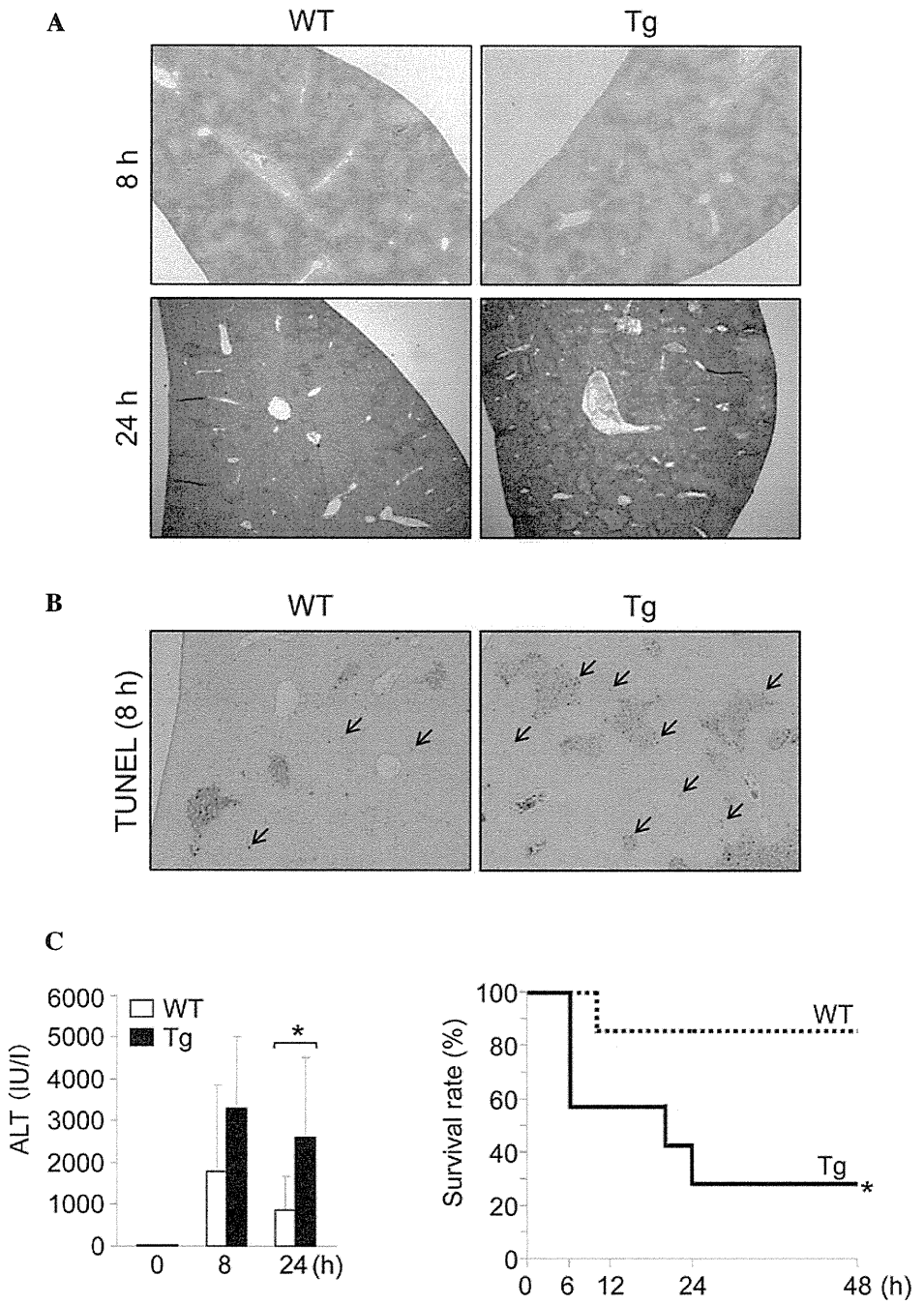

Figure 1. GnT-V Tg mice are sensitive to ConA-induced hepatic injury. (A) Photomicrographs of representative hematoxylin and eosin-stained mouse livers 8 or 24 h after the injection of ConA at 12.5 mg/kg BW (magnification, x40). (B) Photomicrographs of representative TUNEL-stained mouse livers 8 h after the injection of ConA at 12.5 mg/kg BW. Arrows indicate apoptotic hepatocytes (magnification, x40). (C) Serum ALT levels in mice 0 (n=3), 8 (n=8-9) and 24 (n=7-8) h after the injection of ConA at 12.5 mg/kg BW. Results are expressed as the mean ± standard deviation; $^{*}P<0.05$. The survival rate following ConA injection. Mice were injected intravenously with ConA at 20 mg/kg BW and followed for 48 h (n=7); $^{*}P<0.05$. GnT-V, *N*-acetylglucosaminyltransferase V; WT, wild-type; Tg, transgenic; ConA, concanavalin A; BW, body weight; ALT, alanine aminotransferase TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

TUNEL-positive cells (Fig. 1B). The serum ALT levels 24 h after ConA injection were significantly higher in the GnT-V Tg mice compared with those in the WT mice (Fig. 1C). To compare the survival rate of each mouse following ConA administration, a relatively high dose of ConA (20 mg/kg/BW) was injected into each mouse. A significantly higher mortality rate was identified in the GnT-V Tg mice compared with the WT mice (Fig. 1D). In total, >40% of the GnT-V Tg mice succumbed to mortality within 6 h following ConA injection, with a survival rate of 14% observed 48 h after injection. By contrast, a survival rate of 86% was observed in the WT mice after 48 h.

Subsequently, the gene expression of inflammation-associated cytokines and transcription factors in the mouse livers were investigated. No significant differences were identified in the expression levels of the Th1-associated (*Ifn-γ* and *T-bet*) or Th2-associated (*Il-10* and *Gata-3*) genes in the mouse livers prior to or following injection of ConA (Fig. 2A). The serum

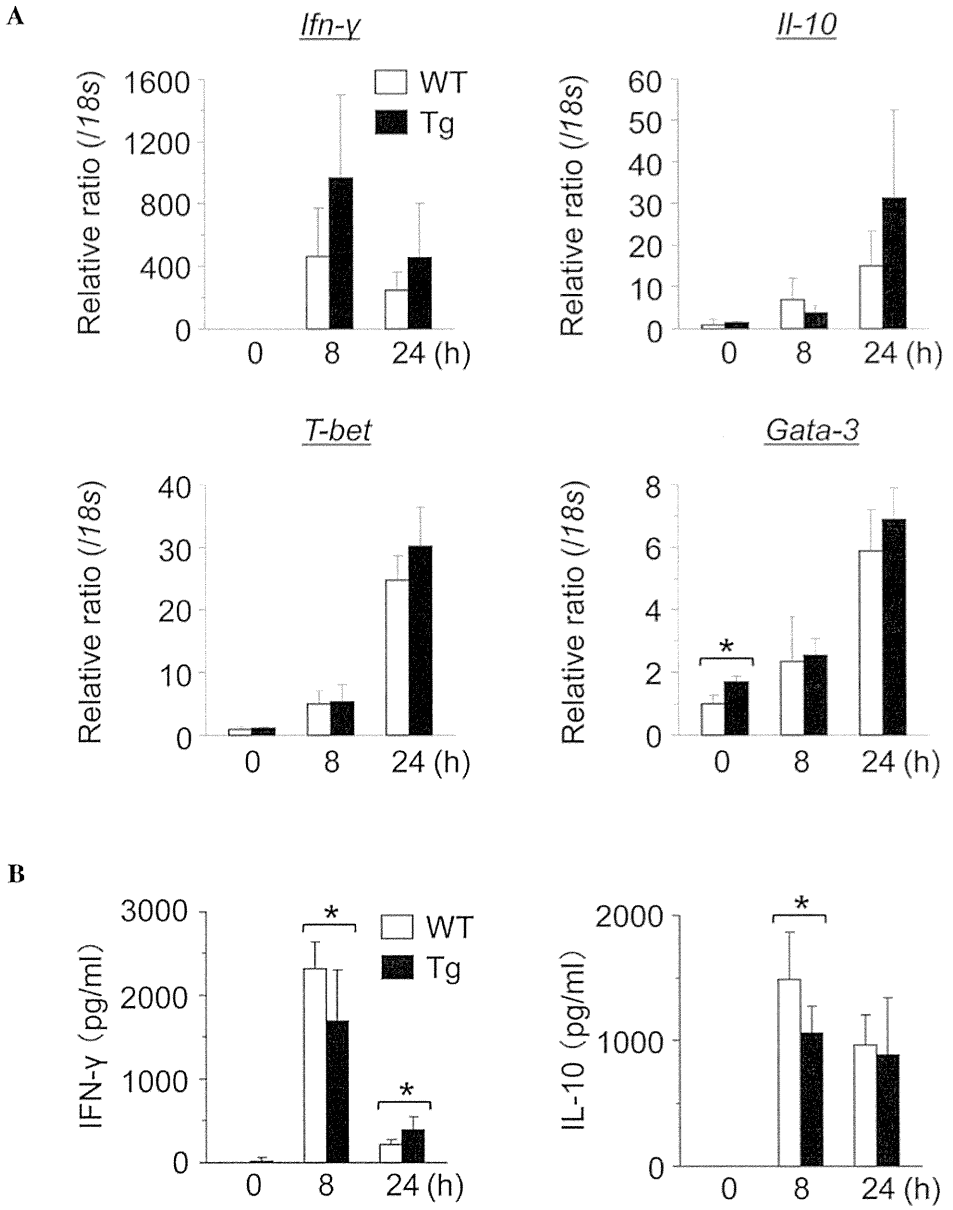

Figure 2. Expression of *in vivo*-activated cytokines demonstrates no significant changes. Hepatic gene expression in WT and GnT-V Tg mice 0, 8, or 24 h after the injection of ConA at 12.5 mg/kg BW. Gene expression of (A) *Ifn-γ*, *Il-10*, *T-bet* and *Gata-3* in mouse livers was evaluated using reverse transcription quantitative polymerase chain reaction. The mRNA expression levels were normalized relative to the mRNA expression of *18s* and are expressed in arbitrary units. The results are expressed as the mean ± standard deviation; $^{*}P<0.05$. (B) Serum cytokine levels in the WT and GnT-V Tg mice 0, 8, or 24 h after the injection of ConA at 12.5 mg/kg BW. Serum levels of IFN-γ and IL-10 were measured by ELISA at the indicated time points. Results are expressed as the mean ± standard deviation; $^{*}P<0.05$. GnT-V, *N*-acetylglucosaminyltransferase V; WT, wild-type; Tg, transgenic; ConA, concanavalin A; BW, body weight; Ifn, interferon; IL, interleukin.

levels of IFN-γ and IL-10 in the GnT-V Tg mice 8 h after the injection of ConA were significantly lower compared with those in the WT mice (Fig. 2B).

Th1 to Th2 cytokine shift in GnT-V Tg mouse splenic lymphocytes in vitro. T cells are important in ConA-induced hepatitis (1,4,5). To investigate function of splenic lymphocytes from the WT and GnT-V Tg mice in cytokine production, the mouse splenic lymphocytes were stimulated with anti-CD3/CD28 antibodies *in vitro*. Levels of the IFN-γ Th1 cytokine were significantly lower in the GnT-V Tg compared with the WT mouse splenic lymphocytes (Fig. 3A), whereas levels of the IL-10 Th2 cytokine were higher in the GnT-V Tg mouse splenic lymphocytes compared with the WT lymphocytes.

ConA activates lymphocyte function, including cytokine production (1,4,5). Subsequently, mouse splenic lymphocytes were stimulated with ConA *in vitro*. As was observed following stimulation with the anti-CD3/CD28 antibodies, the levels of gene expression and the production of IFN-γ in the GnT-V Tg mouse splenic lymphocytes were lower compared with those in the WT, whereas the levels of IL-10 were higher (Fig. 3B and C). IFN-γ is considered a typical pro-inflammatory cytokine (10,11) and IL-10 is known to protect the liver from ConA-induced hepatitis (14,15). Therefore, the exacerbated ConA-induced