operating characteristic curve (AUROC) was calculated for each index. The Youden index was used to identify the optimal cut-off value. Univariate and/or multivariate logistic regression analyses were conducted to identify parameters that significantly contribute to platelet count, NAFLD fibrosis score or mean IMT. Differences were considered statistically significant at $P < 0.05$.

Results

Characteristics of the subjects

The clinical and biochemical characteristics of individuals who participated in this study are shown in Table 1. On average, there was no difference in gender between subjects with and without fatty liver. Age was lower and BMI was higher in fatty liver subjects compared with subjects without fatty liver. Serum levels of T-Bil, CHE, TG, FBG, albumin and Hb were also higher in fatty liver subjects. In contrast, ALT, AST/ALT ratio, HDL-C and adiponectin were lower in fatty liver subjects. The max and mean IMT were somewhat lower in fatty liver subjects. This finding conflicted with previous reports indicating that IMT values are higher in NAFLD patients than in normal subjects (39). Age is an independent determinant of the IMT value (40, 41); therefore, the lower IMT in fatty liver patients in this study could be related to their lower age compared with subjects without fatty liver. There were no differences in platelet count or NAFLD fibrosis score between subjects with fatty liver and those without. These data indicate that the fatty liver subjects in our study did not have progressive vascular and liver fibrosis.

The serum Fuc-Hpt level was measured as a noninvasive biomarker for NASH diagnosis (42). Although there was no significant difference in Fuc-Hpt between the two groups, the mean Fuc-Hpt value was higher in subjects without fatty liver, indicating that our control subjects (those without fatty liver) were not completely healthy individuals. Serum fetuin-A levels were higher in fatty liver subjects than in those without fatty liver, but the difference was not significant.

Table 1. Clinical and serological characteristics of the subjects

Factor	All subjects (n = 295)	Without fatty liver (n = 20)	With fatty liver (n = 275)	P* value
Age (y)	56.7 ± 7.0	61.0 ± 7.0	56.4 ± 6.9	<0.01
Gender (M/F)	164/131	13/7	151/124	0.38
BMI (kg/m^2)	26.2 ± 3.7	22.2 ± 2.6	26.5 ± 3.6	<0.01
sBP level (mmHg)	120.7 ± 15.2	117.3 ± 15.5	121.0 ± 15.2	0.34
AST (U/L)	33.3 ± 23.1	35.3 ± 26.5	33.2 ± 22.8	0.63
ALT (U/L)	46.2 ± 36.1	52.1 ± 99.2	45.8 ± 26.6	<0.01
AST/ALT ratio	0.82 ± 0.31	1.08 ± 0.50	0.80 ± 0.28	<0.01
GGT (U/L)	69.3 ± 101.1	114.7 ± 189.1	66.0 ± 91.2	0.66
T-Bil (mg/dl)	0.76 ± 0.30	0.63 ± 0.17	0.77 ± 0.31	<0.05
CHE (IU/L)	390.8 ± 69.0	347.9 ± 87.7	394.0 ± 66.4	<0.01
T-Chol (mg/dl)	212.5 ± 35.3	207.1 ± 43.2	212.9 ± 34.7	0.35
TG (mg/dl)	153.6 ± 97.3	114.1 ± 87.5	156.5 ± 97.4	<0.01
HDL-C (mg/dl)	54.3 ± 11.1	63.4 ± 17.0	53.6 ± 10.3	<0.01
Creatinine (mg/dl)	0.78 ± 0.19	0.84 ± 0.30	0.77 ± 0.18	0.34
FBG (mg/dl)	121.9 ± 33.5	107.5 ± 16.2	123.0 ± 34.2	<0.05
HbA1c (NGSP) (%)	6.78 ± 1.29	6.2 ± 0.7	6.8 ± 1.3	0.10
Albumin (g/dl)	4.4 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	<0.05
Iron (μg/dl)	112.3 ± 38.0	99.2 ± 40.1	113.4 ± 37.8	0.066
Uric acid (mg/dl)	5.8 ± 1.3	5.5 ± 1.1	5.9 ± 1.3	0.16
Hb (g/dl)	14.3 ± 1.2	13.6 ± 1.1	14.3 ± 1.2	<0.01
Platelet count (×10^9/L)	224.1 ± 54.0	212.0 ± 48.7	225.0 ± 54.3	0.29
NAFLD fibrosis score	-1.37 ± 1.10	-1.21 ± 1.01	-1.38 ± 1.10	0.58
Max IMT (mm)	0.93 ± 0.32	1.31 ± 053	0.91 ± 0.29	<0.01
Mean IMT (mm)	0.85 ± 0.24	1.14 ± 0.41	0.83 ± 0.22	<0.01
Adiponectin (μg/ml)	5.11 ± 2.81	8.12 ± 2.77	4.89 ± 2.69	<0.01
Fuc-Hpt (U/ml)	636.0 ± 1065.1	847.4 ± 927.3	620.2 ± 1074.7	0.22
Fetuin-A (μg/ml)	194.9 ± 32.3	182.9 ± 23.6	195.8 ± 32.7	0.074

Data are presented as the mean ± SD.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CHE, choline esterase; FBG, fasting blood glucose; Fuc-Hpt, fucosylated haptoglobin; GGT, gamma glutamyltranspeptidase; Hb, haemoglobin; IMT, intima media thickness; NAFLD, nonalcoholic fatty liver disease; sBP, systolic blood pressure; T-Bil, total bilirubin; T-Chol, total cholesterol; TG, triglyceride.

*P values correspond to the comparison between without and with fatty liver groups. Wilcoxon test for continuous factors and Pearson's Chi-square test for categorical factors were used.

Relationship between serum fetuin-A, anthropometric parameters and laboratory tests

The results of analyses of Pearson's correlations between serum fetuin-A level and other parameters are summarized in Table 2 and Fig. 1. Serum fetuin-A level was negatively and significantly correlated with age, NAFLD fibrosis score, max IMT and mean IMT. In addition, the serum fetuin-A level was positively and significantly correlated with AST, ALT, GGT and platelet count. The positive correlation between fetuin-A and liver enzymes (AST, ALT and GGT) indicates that fetuin-A promotes liver inflammation. The negative correlation between fetuin-A and NAFLD fibrosis score and the positive correlation between fetuin-A and platelet count indicates that fetuin-A inhibits liver fibrosis. Moreover, the negative correlation between fetuin-A and IMT values indicates that fetuin-A contributes negatively to vascular fibrosis in NAFLD patients.

Relationship between platelet count, anthropometric parameters and laboratory tests

Because advanced liver fibrosis causes portal hypertension and spleen enlargement, thrombocytopaenia has long been known to be accompanied by a degree of liver fibrosis (43–46). We investigated the relationship between platelet count, anthropometric parameters and laboratory tests in NAFLD subjects (Table 3). Univariate analyses revealed that age, AST/ALT ratio, T-Bil, creatinine, iron, Hb and NAFLD fibrosis score had negative relationships, whereas CHE, T-Chol and fetuin-A had positive relationships with the platelet count. We performed multiple logistic regression analyses to determine which variables are significant determinants of the platelet count. Because the platelet count is included in the NAFLD fibrosis score calculation, we omitted the NAFLD fibrosis score from the multiple logistic regression analyses, which demonstrated that age ($P < 0.05$), creatinine ($P < 0.05$), Hb ($P < 0.05$) and serum fetuin-A ($P < 0.05$) levels are significant and independent determinants of the platelet count. Unfortunately, as no spleen diameter data were acquired in our subjects, we could not investigate the relationship between serum fetuin-A and spleen size.

Table 2. Correlation coefficients of relationships between serum fetuin-A levels and various parameters

Factor	*R*	*P* value
Age (y)	−0.21	<0.01
BMI (kg/m^2)	0.17	<0.01
sBP level (mmHg)	0.10	0.084
AST (U/L)	0.23	<0.01
ALT (U/L)	0.27	<0.01
AST/ALT ratio	−0.23	<0.01
GGT (U/L)	0.19	<0.01
T-Bil (mg/dl)	0.031	0.59
CHE (IU/L)	0.19	<0.01
T-Chol (mg/dl)	0.067	0.25
TG (mg/dl)	0.12	<0.05
HDL-C (mg/dl)	−0.027	0.65
Creatinine (mg/dl)	−0.025	0.67
FBG (mg/dl)	−0.012	0.84
HbA1c (NGSP) (%)	−0.071	0.22
Albumin (g/dl)	0.20	<0.01
Iron (μg/dl)	0.18	<0.01
Uric acid (mg/dl)	0.095	0.10
Hb (g/dl)	0.14	<0.05
Platelet count (×10^9/L)	0.19	<0.01
NAFLD fibrosis score	−0.25	<0.01
Max IMT	−0.22	<0.01
Mean IMT	−0.22	<0.01
Adiponectin	−0.11	0.067
Fuc-Hpt	0.058	0.33

Relationship between NAFLD fibrosis score, anthropometric parameters and laboratory tests

The NAFLD fibrosis score is widely used in the noninvasive scoring system to predict the degree of liver fibrosis in NAFLD patients (32). We investigated the relationship between the NAFLD fibrosis score, anthropometric parameters and laboratory tests in NAFLD subjects (Table 4). In univariate analyses, the NAFLD fibrosis score had negative relationships with AST, ALT, GGT, CHE, T-Chol, albumin, platelet count and fetuin-A and positive relationships with age, BMI, AST/ALT ratio, FBG, HbA1c (NGSP) and adiponectin. We omitted age, BMI, AST, ALT, platelet count and albumin from the multiple logistic regression analyses because these variables are considered in the calculation of the NAFLD fibrosis score. The multiple logistic regression analyses demonstrated that T-Chol ($P < 0.01$), adiponectin ($P < 0.05$) and serum fetuin-A levels ($P < 0.01$) were significant and independent determinants of the NAFLD fibrosis score.

Relationship between mean IMT, anthropometric parameters and laboratory tests

Next, we analysed the correlation between the serum fetuin-A level and mean IMT in four groups segmented by mean IMT value quartile (range of mean IMT; group 1: <0.7 mm, group 2: 0.7–0.8 mm, group 3: 0.8–1.0 and group 4: ≥1 mm). Interestingly, the serum fetuin-A level did not change in the three normal mean IMT groups (mean IMT <1 mm) (group 1: 202.3 ± 35.0, group 2: 201.3 ± 31.1 and group 3: 204.0 ± 30.5 μg/ml) but decreased significantly in group 4 (mean IMT ≥1 mm) (180.9 ± 30.1 μg/ml) (Fig. 2A). The AUROC, sensitivity, specificity, PPV and NPV for the prediction of increased mean IMT (≥1 mm) were 0.713%, 66.7%, 74.3%, 46.2%, and 87.1% respectively (Fig. 2B). The serum fetuin-A cut-off value was 183.0 μg/ml.

In addition, we investigated the relationships between mean IMT, anthropometric parameters and

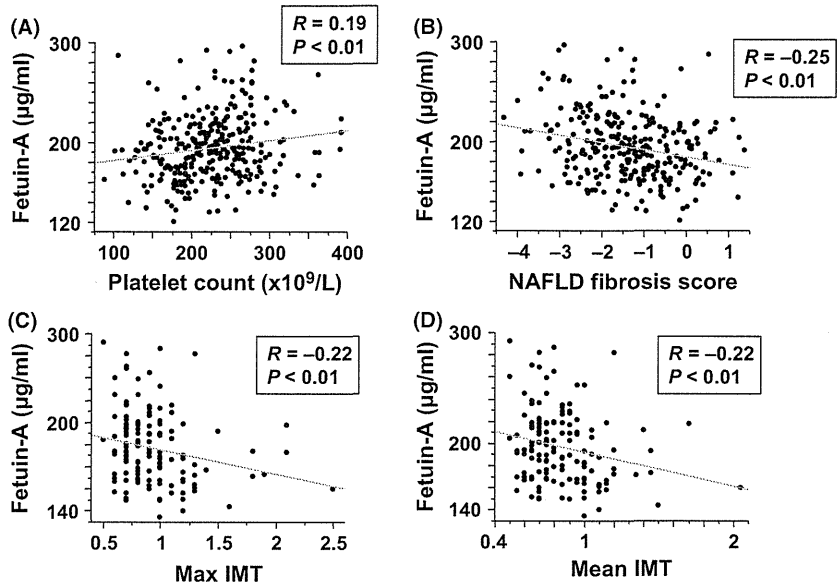

Fig. 1. Relationships between serum fetuin-A level and fibrosis-related factors. Fetuin-A and (A) platelet count, (B) NAFLD fibrosis score, (C) max IMT and (D) mean IMT. R represents the Spearman's correlation coefficient value. NAFLD, nonalcoholic fatty liver disease; IMT, intima media thickness.

Table 3. Univariate and multivariate analysis between platelet count and clinical variables

				95% CI		
Factor	*R*	*P* value	β	Lower	Upper	*P* value
Age (y)	−0.18	<0.01	−1.01	−1.91	−0.11	<0.05
BMI (kg/m^2)	0.10	0.077				
sBP level (mmHg)	−0.0047	0.94				
AST (U/L)	−0.10	0.10				
ALT (U/L)	0.0029	0.96				
AST/ALT ratio	−0.15	<0.05	−18.79	−39.07	1.48	0.069
GGT (U/L)	−0.027	0.65				
T-Bil (mg/dl)	−0.18	<0.01	−13.95	−35.56	7.66	0.20
CHE (IU/L)	0.25	<0.01	0.085	−0.012	0.18	0.086
T-Chol (mg/dl)	0.24	<0.01	0.16	−0.014	0.33	0.072
TG (mg/dl)	−0.085	0.88				
HDL-C (mg/dl)	0.081	0.17				
Creatinine (mg/dl)	−0.24	<0.01	−38.77	−73.39	−4.15	<0.05
FBG (mg/dl)	0.025	0.67				
HbA1c (NGSP) (%)	0.092	0.12				
Albumin (g/dl)	0.045	0.46				
Iron (μg/dl)	−0.13	<0.05	−0.055	−0.23	0.12	0.54
Uric acid (mg/dl)	−0.059	0.31				
Hb (g/dl)	−0.15	<0.05	−7.12	−13.22	−1.02	<0.05
NAFLD fibrosis score	−0.62	<0.01				
Adiponectin (μg/ml)	0.0029	0.96				
Fuc-Hpt (U/ml)	−0.11	0.067				
Fetuin-A (μg/ml)	0.19	<0.01	0.20	0.019	0.39	<0.05

laboratory tests using multiple logistic regression analysis (Table 5). Among the various parameters examined, only the ALT ($P < 0.05$), AST/ALT ratio ($P < 0.01$) and fetuin-A level ($P < 0.05$) were significant and independent determinants of the mean IMT value. We also analysed the relationships between max IMT and other parameters. Multivariate analyses showed that only the AST/ALT ratio was a significant determinant of the max IMT value ($P < 0.05$; data not shown).

Table 4. Univariate and multivariate analysis between NAFLD fibrosis score and clinical variables

Factor	*R*	*P* value	β	95% CI		*P* value
				Lower	Upper	
Age (y)	0.43	<0.01				
BMI (kg/m^2)	0.14	<0.05				
sBP level (mmHg)	0.092	0.13				
AST (U/L)	−0.22	<0.01				
ALT (U/L)	−0.39	<0.01				
AST/ALT ratio	0.45	<0.01				
GGT (U/L)	−0.29	<0.01	−0.00067	−0.0021	0.00081	0.37
T-Bil (mg/dl)	0.024	0.70				
CHE (IU/L)	−0.16	<0.01	−0.0013	−0.0033	0.00074	0.21
T-Chol (mg/dl)	−0.23	<0.01	−0.0059	−0.0096	−0.0022	<0.01
TG (mg/dl)	−0.027	0.66				
HDL-C (mg/dl)	−0.055	0.076				
Creatinine (mg/dl)	0.034	0.58				
FBG (mg/dl)	0.41	<0.01				
HbA1c (NGSP) (%)	0.45	<0.01				
Albumin (g/dl)	−0.33	<0.01				
Iron (μg/dl)	−0.031	0.62				
Uric acid (mg/dl)	−0.066	0.28				
Hb (g/dl)	−0.055	0.37				
Platelet count (×10^9/L)	−0.62	<0.01				
Adiponectin (μg/ml)	0.18	<0.01	0.058	0.014	0.10	<0.05
Fuc-Hpt (U/ml)	0.11	0.074				
Fetuin-A (μg/ml)	−0.25	<0.01	−0.0065	−0.011	−0.0025	<0.01

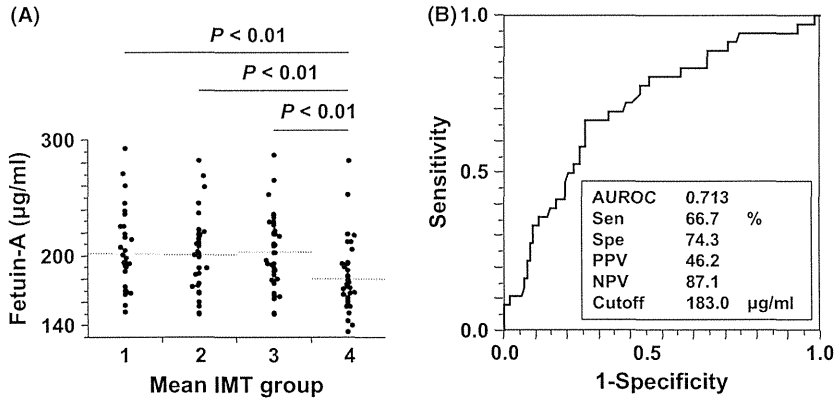

Fig. 2. Serum fetuin-A level in each group, segmented by mean IMT values. Subjects were divided into four groups by quartile of mean IMT value (values of mean IMT; group 1: <0.7 mm, group 2: 0.7–0.8 mm, group 3: 0.8–1.0 and group 4: ≥1 mm). Horizontal grey lines indicate the mean IMT value in each group. ROC curves for fetuin-A for the prediction of increased mean IMT (≥1 mm). AUROC, area under the receiver operating characteristic curve; Sen, sensitivity; Spe, specificity; PPV, positive predictive value; NPV, negative predictive value.

Effects of fetuin-A and TGF-β1 on human hepatic stellate cells

HSCs play a central role in the progression of liver fibrosis (47), and TGF-β1 is the major growth factor involved in liver fibrosis (48). We investigated the effects of human fetuin-A on the HSC line LX-2 with or without TGF-β1 stimulation. We treated LX-2 cells with fetuin-A (15 μM), TGF-β1 (5 ng/ml) or TGF-β1 (5 ng/ml) + fetuin-A (15 μM) and examined fibrosis-related gene expression changes by quantitative real-time RT-PCR (Fig. 3A–C). Treatment of cells with TGF-β1 significantly increased TGF-β1 and collagen Iα1 expression. Treatment with a combination of TGF-β1 and fetuin-A resulted in significant decreases in TGF-β1 and collagen Iα1 levels compared to treatment with TGF-β1 alone. Fetuin-A alone had no significant effect on TGF-β1 and collagen Iα1 gene expression. Interestingly, fetuin-A and TGF-β1 independently upregulated the expression of the TGF-β pseudoreceptor BAMBI, and

Table 5. Multiple logistic regression analysis between mean IMT and clinical variables

Factor	β	95% CI		*P* value
		Lower	Upper	
Age (y)	0.0066	−0.00072	0.014	0.076
BMI (kg/m^2)	−0.0025	−0.020	0.015	0.78
sBP level (mmHg)	0.0017	−0.0015	0.0050	0.30
AST (U/L)	−0.010	−0.021	0.00089	0.071
ALT (U/L)	0.0080	0.00082	0.015	<0.05
AST/ALT ratio	0.56	0.16	0.95	<0.01
GGT (U/L)	−0.00056	−0.0014	0.00026	0.18
T-Bil (mg/dl)	0.050	−0.14	0.24	0.60
CHE (IU/L)	−0.00038	−0.0012	0.00047	0.37
T-Chol (mg/dl)	−0.00052	−0.0023	0.0012	0.56
TG (mg/dl)	−0.00022	−0.00099	0.00055	0.57
HDL-C (mg/dl)	−0.0021	−0.0081	0.0040	0.50
Creatinine (mg/dl)	0.0024	−0.35	0.35	0.99
FBG (mg/dl)	−0.000044	−0.0031	0.0030	0.98
HbA1c (NGSP) (%)	0.0049	−0.070	0.080	0.90
Albumin (g/dl)	−0.0017	−0.26	0.22	0.89
Iron (μg/dl)	−0.000041	−0.0014	0.0013	0.95
Uric acid (mg/dl)	0.0041	−0.038	0.047	0.85
Hb (g/dl)	0.0067	−0.045	0.058	0.80
Platelet count (×10^9/L)	−0.00014	−0.0011	0.00077	0.76
Adiponectin (μg/ml)	−0.0057	−0.025	0.014	0.57
Fuc-Hpt (U/ml)	0.000021	−0.000028	0.000069	0.40
Fetuin-A (μg/ml)	−0.030	−0.055	−0.0084	<0.05

costimulation with fetuin-A and TGF-β1 further upregulated BAMBI expression in LX-2 cells.

Next, we examined the phosphorylation of Smad3 after TGF-β1 stimulation with or without fetuin-A (Fig. 3D). We found that fetuin-A attenuated TGF-β1-induced phosphorylation of Smad3. These data indicate that fetuin-A could suppress TGF-β1 signalling.

Discussion

Our present study involving subjects who received medical health check-ups demonstrated that the serum fetuin-A level is significantly and negatively correlated with both liver and vascular fibrosis in NAFLD. Multivariate analyses revealed that the serum fetuin-A level is an independent determinant of platelet count, NAFLD fibrosis score and mean IMT. In addition, our *in vitro* study demonstrated that fetuin-A blocks TGF-β1-induced fibrogenic signalling. The results of both our human and *in vitro* studies were consistent and indicate that measurement of the serum fetuin-A level is useful for predicting liver and vascular fibrotic changes in NAFLD subjects.

A recent cross-sectional multicenter study reported that type 2 DM affects the severity of liver fibrosis in NAFLD patients (49). In addition, the presence of type 2 DM is an independent risk factor for atherosclerotic diseases (50). In the present study, we demonstrated that the serum fetuin-A concentration decreases with the progression of liver and vascular fibrosis in NAFLD patients (Figs 1 and 2). Decreased serum fetuin-A levels and insulin resistance in NAFLD patients should synergistically accelerate the progression of both liver and vascular fibrogenesis.

NAFLD is a rapidly growing worldwide medical problem because of the ongoing epidemics of obesity and type 2 DM (51). The prevalence of NAFLD in the general population ranges from 15% to 39% (4, 52), and approximately 30% of Japanese adults have NAFLD (5). Recently, Kahraman *et al.* reported that the serum fetuin-A level is significantly higher in NASH patients than in simple steatosis patients (53). Because fetuin-A is a pro-inflammatory protein secreted by hepatocytes, the inflammation in NASH-affected liver would enhance hepatic fetuin-A production. Interestingly, Ballestri *et al.* reported that the serum fetuin-A level is significantly lower in NAFLD patients with CVD than in NAFLD patients without CVD (54). In addition, they found that elevated serum fetuin-A levels are an independent negative predictor of CVD. Their findings were consistent with ours. Both their study and ours indicate that elevated serum fetuin-A levels in NAFLD patients prevent liver and vascular fibrosis as a self-defence reaction (Fig. 4).

In this study, we showed that fetuin-A inhibits TGF-β signalling in LX-2 HSCs, a result which is in agreement with previous reports (27, 55). Interestingly, our study demonstrated that fetuin-A upregulates BAMBI expression. BAMBI is a TGF-β pseudoreceptor and part of a major negative TGF-β signalling feedback mechanism in HSCs (56, 57). To investigate this major negative feedback pathway in HSCs in the presence and absence of fetuin-A, we analysed the expression of BAMBI. However, the mechanism through which fetuin-A upregulates BAMBI remains unknown. Further investigations are needed to elucidate the precise mechanism.

Our study has some limitations. Firstly, the diagnosis of NAFLD was made by ultrasound, and not all subjects received a liver biopsy. Therefore, we could not investigate the relationship between serum fetuin-A levels and the degree of liver fibrosis. In addition, we could not quantitatively estimate liver fat deposition using ultrasonography. Because our study subjects were recruited from health check-ups, liver biopsy was not feasible for all of the subjects because of ethical concerns. In the near future, we will investigate this relationship in biopsy-proven NAFLD patients who were clinically indicated for liver biopsy. Secondly, we did not measure serum fasting insulin levels in our subjects and could not evaluate the precise degree of insulin resistance. We

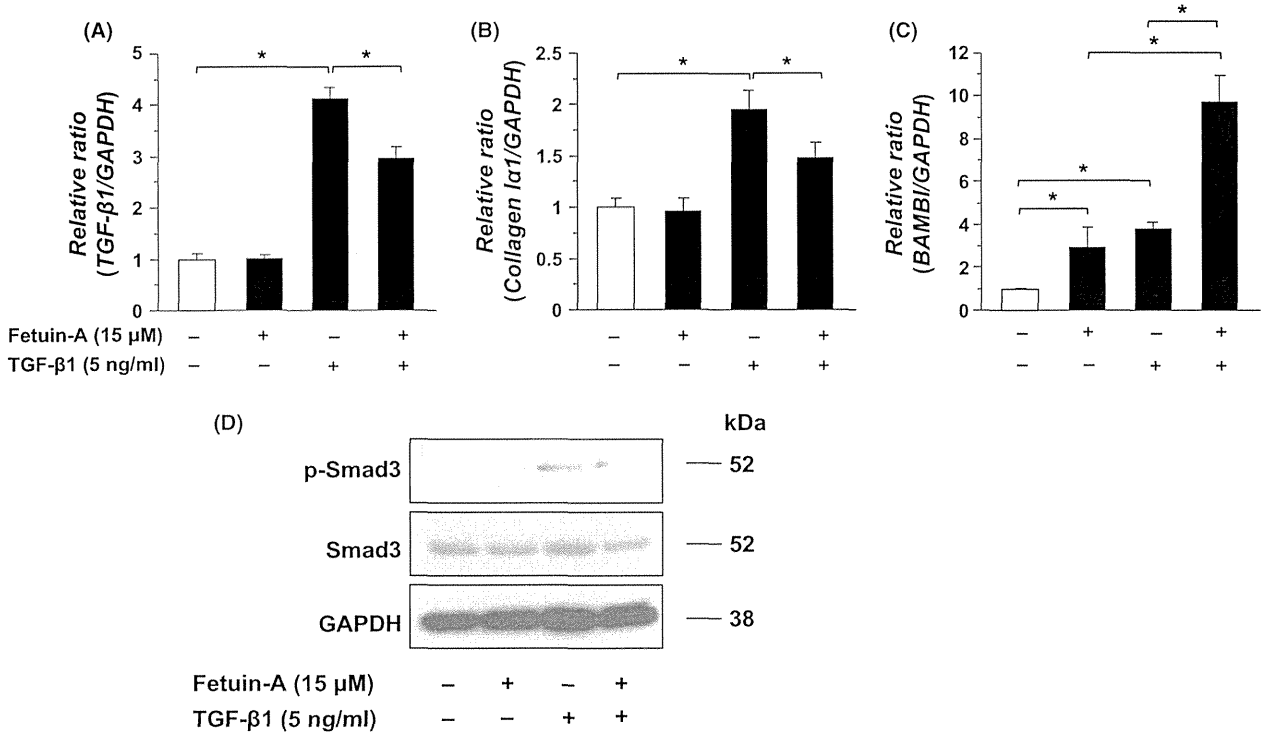

Fig. 3. The effects of fetuin-A and TGF-β1 on human LX-2 HSCs. Levels of (A) TGF-β1, (B) collagen Iα1 and (C) BAMBI gene expression in LX-2 cells with (+) or without (–) fetuin-A (15 μM) and TGF-β1 (5 ng/ml). Levels of gene expression were normalized to GAPDH gene expression and expressed in arbitrary units. Data are means ± SD. $*P < 0.05$ (Wilcoxon test). BAMBI, bone morphogenic protein and activin membrane-bound inhibitor. (D) Immunoblot analysis of p-Smad3, Smad3 and GAPDH. LX-2 cells were incubated for 30 min with (+) or without (–) fetuin-A (15 μM) and TGF-β1 (5 ng/ml). A total of 15 μg of LX-2 whole-cell lysate protein was applied for immunoblotting analysis.

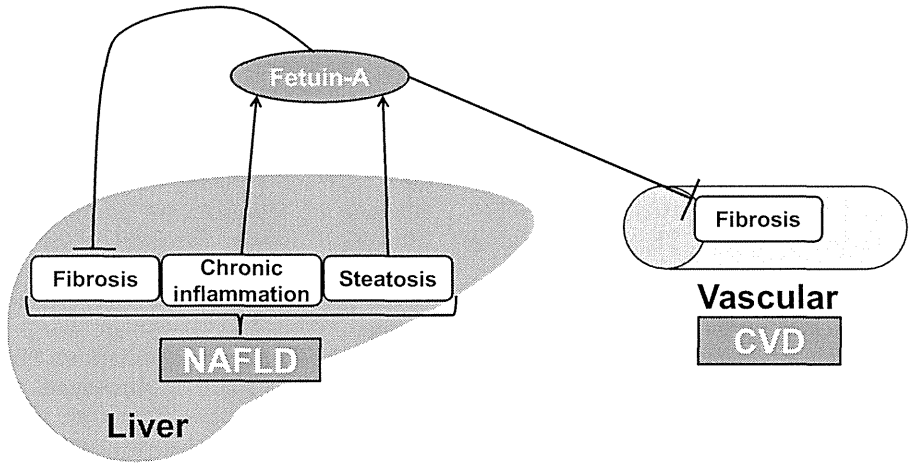

Fig. 4. Putative relationships linking NAFLD, fetuin-A and CVD. We propose that the level of fetuin-A, which increases with chronic inflammatory and fatty changes in the liver, directly suppresses the progression of both liver and vascular fibrosis. CVD, cardiovascular disease.

measured FBG but found no significant relationship between the serum fetuin-A level and FBG. Thirdly, the number of subjects with severe IMT in our study was insufficient to examine the relationship between the serum fetuin-A level and mean IMT precisely in the subjects with vascular fibrosis. Although there was no significant change in the serum fetuin-A level in subjects with normal mean IMT (<1 mm), the level decreased significantly in subjects with abnormal mean IMT (≥1 mm). These results indicate that a decrease in serum fetuin-A level may be a good predictor of the onset of early atherosclerosis. Finally, our study did not

prospectively assess the diagnostic value of fetuin-A in a validation cohort, but we plan to do so in a future study.

In conclusion, measurement of the level of fetuin-A is a noninvasive method, and our results indicate that fetuin-A might prove to be a useful biomarker for predicting liver and vascular fibrosis in NAFLD patients who received health check-ups. Low serum fetuin-A levels are suggestive of enhanced progression of liver and vascular fibrosis in NAFLD patients.

Acknowledgements

The authors would like to thank Akiko Shirai (Research & Development Div., DS Pharma Biomedical Co., Ltd.) for development and technical assistance with the fetuin ELISA kit.

Financial support: This study was supported by Grants-in-Aid for Scientific Research (A), No. 21249038, and (C), No. 24590972, from the Japan Society for the Promotion of Science and by the Global COE Program of Osaka University, funded by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflicts of interests: The authors do not have any disclosures to report.

References

1. Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *JAMA* 2002; **287**: 356–9.
2. Bugianesi E, Leone N, Vanni E, *et al.* Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002; **123**: 134–40.
3. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; **37**: 1202–19.
4. Browning JD, Szczepaniak LS, Dobbins R, *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387–95.
5. Eguchi Y, Hyogo H, Ono M, *et al.* Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. *J Gastroenterol* 2012; **47**: 586–95.
6. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221–31.
7. Liou I, Kowdley KV. Natural history of nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2006; **40**(Suppl. 1): S11–6.
8. Nascimbeni F, Pais R, Bellentani S, *et al.* From NAFLD in clinical practice to answers from guidelines. *J Hepatol* 2013; **59**: 859–71.
9. Vanni E, Bugianesi E, Kotronen A, *et al.* From the metabolic syndrome to NAFLD or vice versa? *Dig Liver Dis* 2010; **42**: 320–30.
10. Anstee QM, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 330–44.
11. Ou HY, Wang CY, Yang YC, Chen MF, Chang CJ. The association between nonalcoholic fatty pancreas disease and diabetes. *PLoS ONE* 2013; **8**: e62561.
12. Oni ET, Agatston AS, Blaha MJ, *et al.* A systematic review: burden and severity of subclinical cardiovascular disease among those with nonalcoholic fatty liver; should we care? *Atherosclerosis* 2013; **230**: 258–67.
13. Villanova N, Moscatiello S, Ramilli S, *et al.* Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 473–80.
14. Salvi P, Ruffini R, Agnoletti D, *et al.* Increased arterial stiffness in nonalcoholic fatty liver disease: the Cardio-GOOSE study. *J Hypertens* 2010; **28**: 1699–707.
15. Targher G, Bertolini L, Padovani R, *et al.* Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2007; **30**: 1212–8.
16. Lonardo A, Sookoian S, Chonchol M, Loria P, Targher G. Cardiovascular and systemic risk in nonalcoholic fatty liver disease - atherosclerosis as a major player in the natural course of NAFLD. *Curr Pharm Des* 2013; **19**: 5177–92.
17. Kamada Y, Takehara T, Hayashi N. Adipocytokines and liver disease. *J Gastroenterol* 2008; **43**: 811–22.
18. Stefan N, Kantartzis K, Haring HU. Causes and metabolic consequences of Fatty liver. *Endocr Rev* 2008; **29**: 939–60.
19. Tonjes A, Fasshauer M, Kratzsch J, Stumvoll M, Bluher M. Adipokine pattern in subjects with impaired fasting glucose and impaired glucose tolerance in comparison to normal glucose tolerance and diabetes. *PLoS ONE* 2010; **5**: e13911.
20. Denecke B, Graber S, Schafer C, *et al.* Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. *Biochem J* 2003; **376**(Pt. 1): 135–45.
21. Srinivas PR, Wagner AS, Reddy LV, *et al.* Serum alpha 2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. *Mol Endocrinol* 1993; **7**: 1445–55.
22. Mathews ST, Singh GP, Ranalletta M, *et al.* Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene. *Diabetes* 2002; **51**: 2450–8.
23. Pal D, Dasgupta S, Kundu R, *et al.* Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med* 2012; **18**: 1279–85.
24. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002; **7**: d793–807.
25. Toma I, Mccaffrey TA. Transforming growth factor-beta and atherosclerosis: interwoven atherogenic and atheroprotective aspects. *Cell Tissue Res* 2012; **347**: 155–75.
26. Rittenberg B, Partridge E, Baker G, *et al.* Regulation of BMP-induced ectopic bone formation by Ahsg. *J Orthop Res* 2005; **23**: 653–62.
27. Verma-Gandhu M, Peterson MR, Peterson TC. Effect of fetuin, a TGFbeta antagonist and pentoxifylline, a cytokine antagonist on hepatic stellate cell function and fibrotic parameters in fibrosis. *Eur J Pharmacol* 2007; **572**: 220–7.
28. Merx MW, Schafer C, Westenfeld R, *et al.* Myocardial stiffness, cardiac remodeling, and diastolic dysfunction in calcification-prone fetuin-A-deficient mice. *J Am Soc Nephrol* 2005; **16**: 3357–64.
29. Guillory B, Sakwe AM, Saria M, *et al.* Lack of fetuin-A (alpha2-HS-glycoprotein) reduces mammary tumor inci-

dence and prolongs tumor latency via the transforming growth factor-beta signaling pathway in a mouse model of breast cancer. *Am J Pathol* 2010; **177**: 2635–44.
30. Saadeh S, Younossi ZM, Remer EM, *et al.* The utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 745–50.
31. Hamano M, Kamada Y, Kiso S, *et al.* Adiponectin negatively correlates with alcoholic and non-alcoholic liver dysfunction: health check-up study of Japanese men. *Hepatol Res* 2013; **43**: 238–48.
32. Angulo P, Hui JM, Marchesini G, *et al.* The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846–54.
33. Hirata A, Kishida K, Hiuge-Shimizu A, *et al.* Qualitative score of systemic arteriosclerosis by vascular ultrasonography as a predictor of coronary artery disease in type 2 diabetes. *Atherosclerosis* 2011; **219**: 623–9.
34. Kuwamoto K, Takeda Y, Shirai A, *et al.* Identification of various types of alpha2-HS glycoprotein in sera of patients with pancreatic cancer: possible implication in resistance to protease treatment. *Mol Med Rep* 2010; **3**: 651–6.
35. Kamada Y, Kinoshita N, Tsuchiya Y, *et al.* Reevaluation of a lectin antibody ELISA kit for measuring fucosylated haptoglobin in various conditions. *Clin Chim Acta* 2013; **417**: 48–53.
36. Kumada M, Kihara S, Sumitsuji S, *et al.* Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol* 2003; **23**: 85–9.
37. Xu L, Hui AY, Albanis E, *et al.* Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 2005; **54**: 142–51.
38. Kamada Y, Mori K, Matsumoto H, *et al.* N-Acetylglucosaminyltransferase V regulates TGF-beta response in hepatic stellate cells and the progression of steatohepatitis. *Glycobiology* 2012; **22**: 778–87.
39. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; **49**: 600–7.
40. Aminbakhsh A, Mancini GB. Carotid intima-media thickness measurements: what defines an abnormality? A systematic review. *Clin Invest Med* 1999; **22**: 149–57.
41. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. *Circulation* 2007; **115**: 459–67.
42. Kamada Y, Akita M, Takeda Y, *et al.* Serum Fucosylated Haptoglobin as a Novel Diagnostic Biomarker for Predicting Hepatocyte Ballooning and Nonalcoholic Steatohepatitis. *PLoS ONE* 2013; **8**: e66328.
43. Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Hepatology* 1988; **8**: 493–6.
44. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003; **362**: 2095–100.
45. Udell JA, Wang CS, Tinmouth J, *et al.* Does this patient with liver disease have cirrhosis? *JAMA* 2012; **307**: 832–42.
46. Cozzolino G, Lonardo A, Francica G, Amendola F, Cacciatore L. Differential diagnosis between hepatic cirrhosis and chronic active hepatitis: specificity and sensitivity of physical and laboratory findings in a series from the Mediterranean area. *Am J Gastroenterol* 1983; **78**: 442–5.
47. Friedman SL. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* 1993; **328**: 1828–35.
48. Bedossa P, Peltier E, Terris B, Franco D, Poynard T. Transforming growth factor-beta 1 (TGF-beta 1) and TGF-beta 1 receptors in normal, cirrhotic, and neoplastic human livers. *Hepatology* 1995; **21**: 760–6.
49. Nakahara T, Hyogo H, Yoneda M, *et al.* Type 2 diabetes mellitus is associated with the fibrosis severity in patients with nonalcoholic fatty liver disease in a large retrospective cohort of Japanese patients. *J Gastroenterol* 2014. (in press).
50. Milicevic Z, Raz I, Beattie SD, *et al.* Natural history of cardiovascular disease in patients with diabetes: role of hyperglycemia. *Diabetes Care* 2008; **31**(Suppl. 2): S155–60.
51. Charlton M. Nonalcoholic fatty liver disease: a review of current understanding and future impact. *Clin Gastroenterol Hepatol* 2004; **2**: 1048–58.
52. Powell EE, Cooksley WG, Hanson R, *et al.* The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; **11**: 74–80.
53. Kahraman A, Sowa JP, Schlattjan M, *et al.* Fetuin-A mRNA expression is elevated in NASH compared with NAFL patients. *Clin Sci (Lond)* 2013; **125**: 391–400.
54. Ballestri S, Meschiari E, Baldelli E, *et al.* Relationship of serum fetuin-a levels with coronary atherosclerotic burden and NAFLD in patients undergoing elective coronary angiography. *Metab Syndr Relat Disord* 2013; **11**: 289–95.
55. Demetriou M, Binkert C, Sukhu B, Tenenbaum HC, Dennis JW. Fetuin/alpha2-HS glycoprotein is a transforming growth factor-beta type II receptor mimic and cytokine antagonist. *J Biol Chem.* 1996; **271**: 12755–61.
56. Seki E, De Minicis S, Osterreicher CH, *et al.* TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007; **13**: 1324–32.
57. Sekiya T, Oda T, Matsuura K, Akiyama T. Transcriptional regulation of the TGF-beta pseudoreceptor BAMBI by TGF-beta signaling. *Biochem Biophys Res Commun* 2004; **320**: 680–4.

Hitomi Asazawa[a], Yoshihiro Kamada[a], Yuri Takeda, Shinji Takamatsu, Shinichiro Shinzaki, Youkoku Kim, Riichiro Nezu, Noriyoshi Kuzushita, Eiji Mita, Michio Kato and Eiji Miyoshi*

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).

DOI 10.1515/cclm-2014-0427
Received April 19, 2014; accepted June 30, 2014

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

[a]Hitomi Asazawa and Yoshihiro Kamada contributed equally to this study.

***Corresponding author: Eiji Miyoshi,** MD, PhD, Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, 1-7, Yamada-oka, Suita, Osaka 565-0871, Japan, Phone/Fax: +81 6 68792590, E-mail: emiyoshi@sahs.med.oska-u.ac.jp
Hitomi Asazawa, Yoshihiro Kamada, Yuri Takeda, Shinji Takamatsu, Shinichiro Shinzaki and Eiji Miyoshi: Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, Osaka, Japan
Youkoku Kim and Riichiro Nezu: Department of Surgery, Osaka Rosai Hospital, Osaka, Japan
Noriyoshi Kuzushita, Eiji Mita and Michio Kato: Department of Hepato-Gastroenterology, Osaka National Hospital, Osaka, Japan

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.

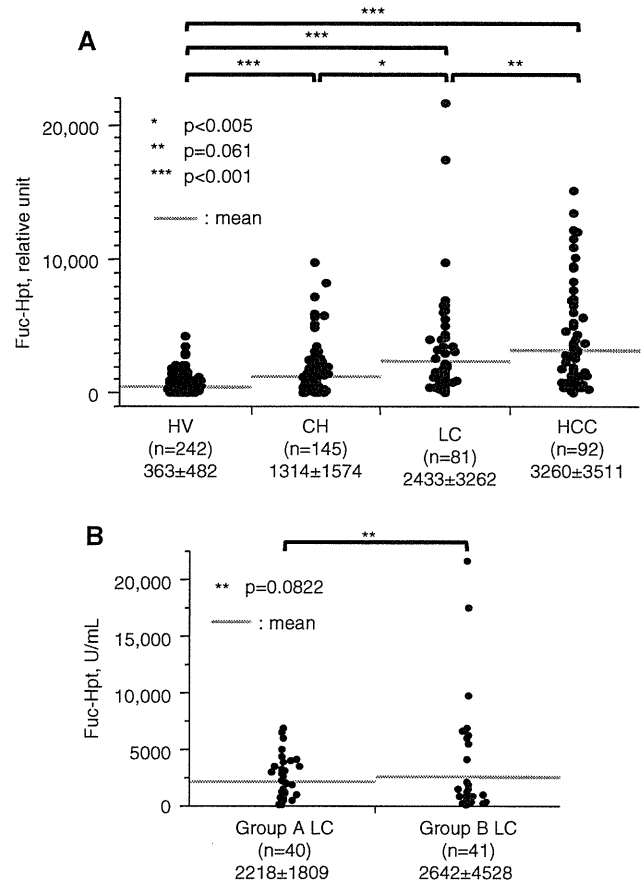

Figure 1 Levels of serum Fuc-Hpt in patients with chronic liver diseases.
(A) Fuc-Hpt levels were measured with a lectin antibody ELISA kit. Values are shown in relative units. (B) Patients with LC were divided into two groups. LC patients in the non-HCC group did not develop HCC within a 5-year period. 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

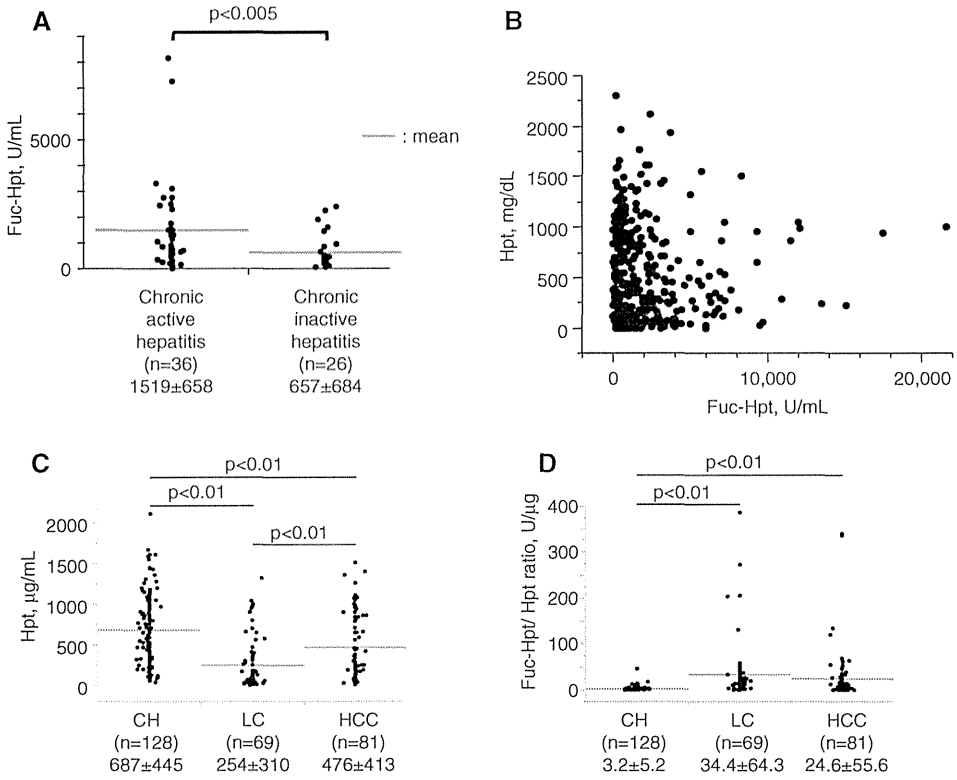

Figure 2 Comparison of serum Fuc-Hpt levels in CH and LC.
(A) Serum Fuc-Hpt levels in patients with chronic active and inactive hepatitis. (B) Fuc-Hpt and total Hpt levels were compared in 318 patients with chronic liver diseases. (C) Serum haptoglobin (Hpt) levels were measured using an ELISA kit in 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.

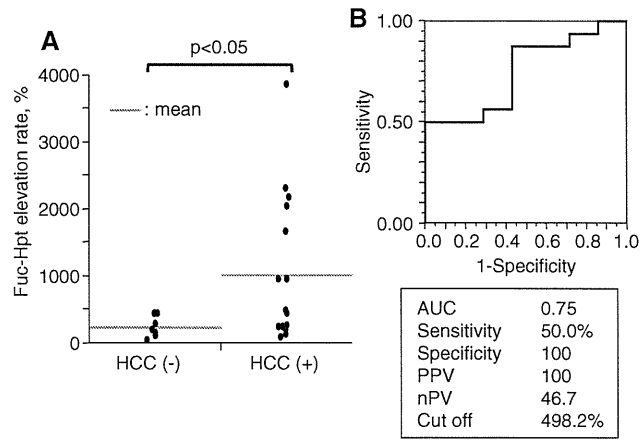

Figure 3 Serum Fuc-Hpt levels were compared with LC alone and LC plus HCC in LC–HCC follow-up patients.
(A) Fuc-Hpt elevation rates were measured in 19 patients with HCC before and after HCC development. Gray bars indicate mean values.
(B) ROC analysis of data in panel A. AUC, area under the curve; NPV,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

Acknowledgments: Financial support: This study was supported by a Grant-in-Aid for Scientific Research (A), No. 21249038 from the Japan Society for the Promotion of Science and performed as a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct), Ministry of Education, Culture, Sports, and Science.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Financial Support: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Miyoshi E, Moriwaki K, Nakagawa T. Biological function of fucosylation in cancer biology. J Biochem 2008;143:725–9.
2. Wang M, Long RE, Comunale MA, Junaidi O, Marrero J, Di Bisceglie AM, et al. Novel fucosylated biomarkers for the early detection of hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev 2009;18:1914–21.
3. Hashimoto S, Asao T, Takahashi J, Yagihashi Y, Nishimura T, Saniabadi AR, et al. alpha1-acid glycoprotein fucosylation as a marker of carcinoma progression and prognosis. Cancer 2004;101:2825–36.
4. Debruyne EN, Vanderschaeghe D, Van Vlierberghe H, Vanhecke A, Callewaert N, Delanghe JR. Diagnostic value of the hemopexin N-glycan profile in hepatocellular carcinoma patients. Clin Chem 2010;56:823–31.
5. Taketa K. Alpha-fetoprotein: reevaluation in hepatology. Hepatology 1990;12:1420–32.
6. Breborowicz J, Mackiewicz A, Breborowicz D. Microheterogeneity of alpha-fetoprotein in patient serum as demonstrated by lectin affino-electrophoresis. Scand J Immunol 1981;14:15–20.
7. Taketa K, Izumi M, Ichikawa E. Distinct molecular species of human alpha-fetoprotein due to differential affinities to lectins. Ann N Y Acad Sci 1983;417:61–8.
8. Aoyagi Y. Carbohydrate-based measurements on alpha-fetoprotein in the early diagnosis of hepatocellular carcinoma. Glycoconj J 1995;12:194–9.
9. Marrero JA, Feng Z, Wang Y, Nguyen MH, Befeler AS, Roberts LR, et al. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. Gastroenterology 2009;137:110–8.
10. Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K, et al. Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. Int J Cancer 2006;118:2803–8.
11. Nakano M, Nakagawa T, Ito T, Kitada T, Hijioka T, Kasahara A, et al. Site-specific analysis of N-glycans on haptoglobin in sera of patients with pancreatic cancer: a novel approach for the development of tumor markers. Int J Cancer 2008;122:2301–9.
12. Takeda Y, Shinzaki S, Okudo K, Moriwaki K, Murata K, Miyoshi E. Fucosylated haptoglobin is a novel type of cancer biomarker linked to the prognosis after an operation in colorectal cancer. Cancer 2012;118:3036–43.
13. Narisada M, Kawamoto S, Kuwamoto K, Moriwaki K, Nakagawa T, Matsumoto H, et al. Identification of an inducible factor secreted by pancreatic cancer cell lines that stimulates the production of fucosylated haptoglobin in hepatoma cells. Biochem Biophys Res Commun 2008;377:792–6.

14. Matsumoto H, Shinzaki S, Narisada M, Kawamoto S, Kuwamoto K, Moriwaki K, et al. Clinical application of a lectin-antibody ELISA to measure fucosylated haptoglobin in sera of patients with pancreatic cancer. Clin Chem Lab Med 2010;48:505–12.
15. Kormoczi GF, Saemann MD, Buchta C, Peck-Radosavljevic M, Mayr WR, Schwartz DW, et al. Influence of clinical factors on the haemolysis marker haptoglobin. Eur J Clin Invest 2006;36: 202–9.
16. Kamada Y, Kinoshita N, Tsuchiya Y, Kobayashi K, Fujii H, Terao N, et al. Reevaluation of a lectin antibody ELISA kit for measuring fucosylated haptoglobin in various conditions. Clin Chim Acta 2013;417:48–53.
17. Kagebayashi C, Yamaguchi I, Akinaga A, Kitano H, Yokoyama K, Satomura M, et al. Automated immunoassay system for AFP-L3% using on-chip electrokinetic reaction and separation by affinity electrophoresis. Anal Biochem 2009;388:306–11.
18. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 1996;24:289–93.
19. Nakagawa T, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T, et al. Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. J Biol Chem 2006;281:29797–806.
20. Nakagawa T, Takeishi S, Kameyama A, Yagi H, Yoshioka T, Moriwaki K, et al. Glycomic analyses of glycoproteins in bile and serum during rat hepatocarcinogenesis. J Proteome Res 2010;9:4888–96.

Supplemental Material: The online version of this article (DOI: 10.1515/cclm-2014-0427) offers supplementary material, available to authorized users.

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

YOSHIHIRO KAMADA[1,2*], MOTOYA SATO[1*], SACHIHO KIDA[1], MAAYA AKITA[1], KAYO MIZUTANI[1], HIRONOBU FUJII[1], TOMOAKI SOBAJIMA[1], YUICHI YOSHIDA[2], SHINICHIRO SHINZAKI[1,2], SHINJI TAKAMATSU[1], TETSUO TAKEHARA[2] and EIJI MIYOSHI[1]

Departments of [1]Molecular Biochemistry and Clinical Investigation; and [2]Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

Received July 3, 2014; Accepted December 9, 2014

DOI: 10.3892/mmr.2015.3168

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.

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

Correspondence to: Professor Eiji Miyoshi, Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, 1-7 Yamada-oka, Suita, Osaka 565-0871, Japan
E-mail: emiyoshi@sahs.med.osaka-u.ac.jp

*Contributed equally

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

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

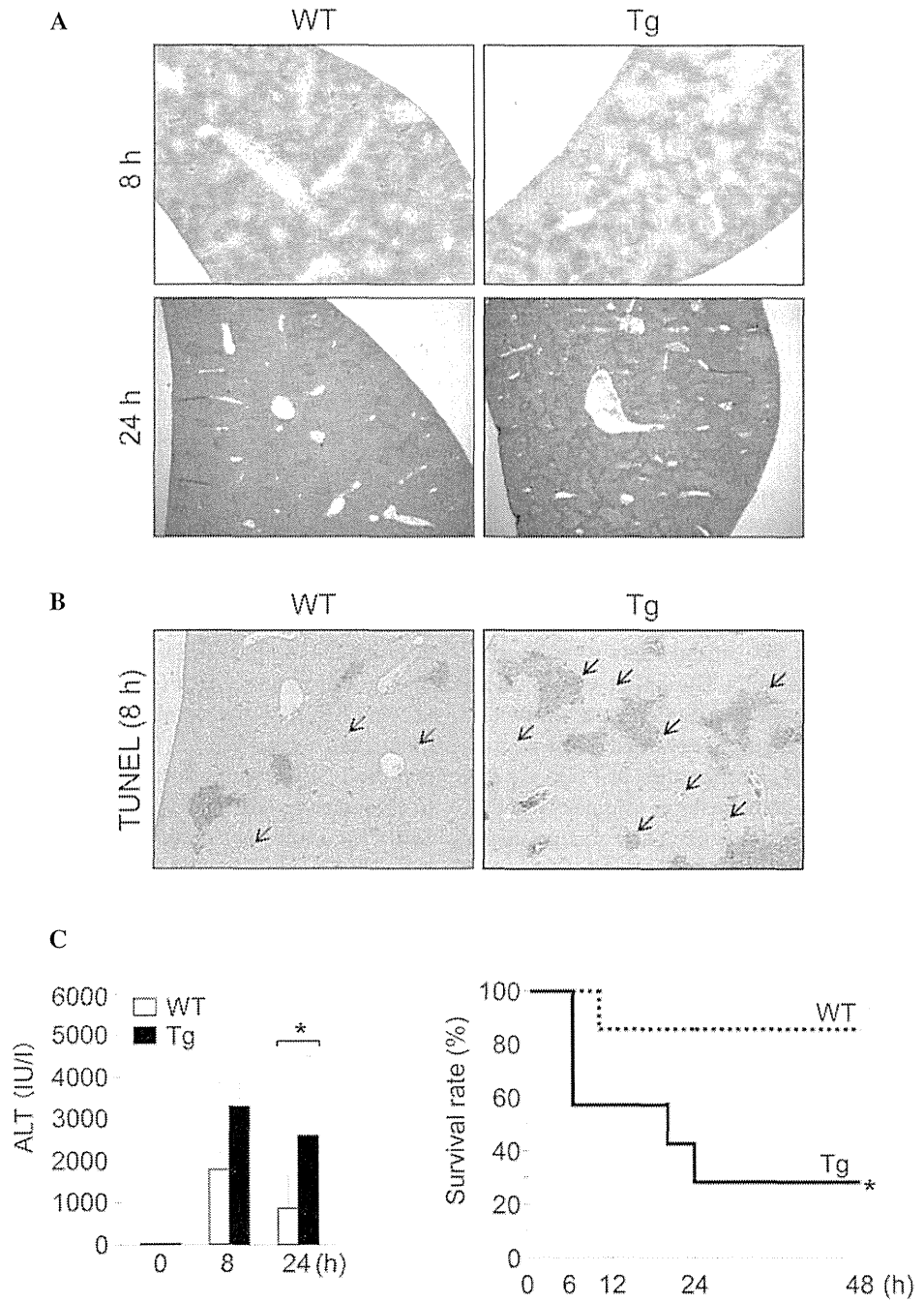

Figure 1. GnT-V Tg mice are sensitive to ConA-induced hepatic injury. (A) Photomicrographs of representative hematoxylin and eosin-stained mouse livers 8 or 24 h after the injection of ConA at 12.5 mg/kg BW (magnification, x40). (B) Photomicrographs of representative TUNEL-stained mouse livers 8 h after 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