FIGURE 3. Pathological evaluation of pancreatic fibrosis. A and B represent grade 0 or 1 (<10% of fibrotic change), C and D represent grade 2 or 3 (11%–30% of [moderate] fibrotic change), and E and F represent grade 4 (>31% of [severe] fibrotic change). A, C, and E show the pancreas region of patients with PDAC, and B, D, and F show the pancreas region of non-PDAC patients. Each scale bar in the figure shows 500 μm.

clinical factors and pancreatic conditions such as fibrosis, fatty degeneration, and inflammatory cell infiltration are listed in Table 1. The ratio of inflammatory cell infiltration was slightly higher in men than in women ($P = 0.045$), and the serum elastase value was higher in patients with inflammatory cell infiltration of the pancreas than in patients without inflammation ($P = 0.003$). No correlation was found between other clinical factors and pancreatic conditions. The presence or absence of neoadjuvant CRT did not affect the pancreatic conditions in PDAC patients (Supplementary Figure 1, http://links.lww.com/MPA/A304).

Comparison Between Normal Pancreatic Samples and Clinical Parameters

Forty-three (44%) of the 98 cases showed positive fatty changes, 3 (3%) showed positive inflammatory changes, and 41 (41.8%) showed positive fibrotic changes. The correlations between clinical factors and pancreatic conditions such as fibrosis, fatty degeneration, and inflammatory cell infiltration are listed in Table 2. The ratio of fibrosis was slightly higher in men than in women ($P = 0.032$). No correlations were found between other clinical factors and pancreatic conditions.

Comparison Between Noncancerous Regions of the Pancreas From Patients With PDAC and Normal Pancreas Specimens

Pancreas specimens from noncancerous regions showed a higher rate of positive changes in fatty degeneration, inflammatory

cell infiltration, and fibrosis than did specimens from non-PDAC patients (Table 3). Interestingly, both pancreatic fatty degeneration and pancreatic fibrosis were significantly higher in patients with PDAC than in control patients. Although the rate of pancreatic inflammation as judged by lymphocyte inflammation was less than 20% in both types of cases, the ratio was much higher in patients with PDAC than in non-PDAC patients. The positive rate of fatty degeneration was significantly correlated with the positive fibrosis rate only in control samples, indicating that fibrotic changes in the pancreas were affected by fatty degeneration in the pancreas in control patients (Fig. 4).

Univariate and Multivariate Analysis of the Primary/Secondary Outcome

To identify parameters that significantly contributed to the primary and secondary outcomes, we performed univariate and multivariate analyses in this study. First, we performed multivariate analysis to explore determinants of the primary outcome (presence of PDAC). Plausible predictors (age, sex, histological changes [fibrosis, fatty degeneration, inflammatory cell infiltration], Brinkman index, weekly ethanol intake, and body mass index [BMI]) were included in this analysis (Table 4). The CRT was not included in these predictors, because CRT was not performed in control patients without PDAC. We found that each type of histological change (fibrosis, fatty degeneration, inflammatory cell infiltration) and the BMI were independent and significant determinants. The BMI was a negative determinant. The

TABLE 1. Correlation Between Clinical Factors and Pancreatic Fibrosis, Fatty Degeneration, and Inflammatory Cell Infiltration in Patients With Pancreatic Cancer

		Patients, n	Fibrosis			Fatty Degeneration			Inflammatory Cell Infiltration		
			Negative	Positive	P	Negative	Positive	P	Negative	Positive	P
Sex	Male	41	6	35	0.97	12	29	0.73	32	9	0.045
	Female	35	5	30		9	26		33	2	
Age, y			67.6 ± 10.2	64.3 ± 10.6	0.34	62.5 ± 10.8	65.6 ± 10.4	0.22	64.6 ± 10.7	65.8 ± 9.8	0.98
Site of tumor	Head	37	4	33	0.55	7	30	0.25	31	6	0.92
	Body	32	5	27		12	20		28	4	
	Tail	8	2	6		3	5		7	1	
Tumor size, mm			22.1 ± 18.8	21.5 ± 10.7	0.6	24.9 ± 13.6	20.5 ± 11.4	0.2	21.7 ± 12.0	21.0 ± 13.0	0.54
Neoadjuvant CRT	Performed	41	5	36	0.43	11	30	0.48	36	5	0.43
	Not performed	26	5	21		5	21		21	5	
HbA1c, %			6.46 ± 1.49	6.69 ± 1.77	0.87	7.05 ± 2.21	6.51 ± 1.54	0.57	6.71 ± 1.77	6.16 ± 1.12	0.67
Glucose, mg/dL			117.5 ± 36.0	128.6 ± 55.1	0.88	134.5 ± 32.6	126.4 ± 57.9	0.41	135.0 ± 51.3	78.8 ± 49.6	0.087
Amylase, IU/L			86.0 ± 28.1	166.5 ± 272.6	0.21	210.1 ± 436.8	131.5 ± 98.8	0.86	158.3 ± 273.9	142.1 ± 104.1	0.58
Pancreatic amylase, IU/L			108.5 ± 119.5	53.0 ± 46.1	0.37	80.3 ± 58.1	53.2 ± 59.8	0.49	61.1 ± 60.4	64.0 ± 65.1	0.77
Elastase, ng/dL			206.3 ± 281.9	471.1 ± 455.2	0.26	441.8 ± 467.5	423.5 ± 442.5	1	287.9 ± 220.9	959.3 ± 668.0	0.057
CA19-9, U/mL			970.9 ± 1993.8	488.8 ± 1685.8	0.89	201.0 ± 278.2	681.2 ± 1982.3	0.96	560.3 ± 1776	510.4 ± 940.1	0.62
Dupan-2, U/mL			210	538.6 ± 1000.8	1	205.8 ± 146.1	649.8 ± 1142.0	0.64	519.2 ± 972.3		
BMI			21.03 ± 2.94	20.22 ± 2.77	0.52	20.27 ± 2.47	20.35 ± 2.92	0.93	20.07 ± 2.62	21.72 ± 3.38	0.1
DM*	(+)	31	28	3	0.32	8	23	0.77	3	28	0.32
	(−)	45	37	8		13	32		8	37	
Brinkman Index†			362.8 ± 681.0	483.9 ± 509.7	0.43	407.2 ± 461.1	491.0 ± 566.8	0.79	447.5 ± 554.9	565.0 ± 412.1	0.36
Ethanol intake, g/w			158.9 ± 269.8	135.9 ± 308.9	0.39	125.0 ± 288.7	145.6 ± 310.3	0.9	135.2 ± 286.6	160.5 ± 388.1	0.61
Fibrosis	Negative	11	−	−		2	9	0.45	11	0	0.14
	Positive	65	−	−		19	46		54	11	
Fatty degeneration	Negative	21	2	19	0.45	−	−		18	4	0.48
	Positive	55	9	46		−	−		48	7	
Inflammatory cell infiltration	Negative	65	11	54	0.14	17	48	0.48	−	−	
	Positive	11	0	11		4	7		−	−	

*The presence of DM was defined as fasting blood glucose of 126 mg/dL or greater, HbA1c (NGSP) of 6.5% or greater, or treatment with antidiabetic drugs.
†The Brinkman index is calculated as the number of cigarettes smoked per day multiplied by the number of years that the participant smoked.

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TABLE 2. Correlation Between Clinical Factors and Pancreatic Fibrosis, Fatty Degeneration, and Inflammatory Cell Infiltration in Non-PDAC Patients

		Patients, n	Fibrosis			Fatty Degeneration			Inflammatory Cell Infiltration		
			Negative	Positive	P	Negative	Positive	P	Negative	Positive	P
Sex	Male	62	31	31	0.032	35	27	0.93	60	2	0.9
	Female	36	26	10		20	16		35	1	
Age, y			64.1 ± 12.0	62.6 ± 13.9	0.61	61.3 ± 13.4	66.3 ± 11.5	0.09	63.5 ± 12.8	60.7 ± 15.6	0.76
Glucose, mg/dL			99.0 ± 30.5	114.4 ± 55.0	0.75	107.0 ± 44.9	101.6 ± 36.6	0.75			
Amylase, IU/L			81.2 ± 45.3	188.6 ± 193.9	0.052	79.5 ± 52.1	159.2 ± 168.7	0.063			
BMI			22.91 ± 4.30	22.66 ± 3.23	0.96	22.54 ± 3.52	23.09 ± 4.21	0.56	22.87 ± 3.82	17.84	0.15
DM*	(+)	13	5	8	0.79	6	7	0.86	0	13	0.49
	(−)	85	36	49		37	48		3	82	
Brinkman Index†			407.4 ± 602.1	482.0 ± 551.6	0.54	455.0 ± 591.4	396.1 ± 583.6	0.58	418.4 ± 585.1	800	0.38
Ethanol intake, g/w			80.7 ± 182.0	124.6 ± 161.1	0.17	121.4 ± 196.6	65.6 ± 140.0	0.19	93.3 ± 175.1	210	0.22
Fibrosis	Negative	57	−	−		37	20	0.039	56	1	0.38
	Positive	41	−	−		18	23		39	2	
Fatty degeneration	Negative	55	37	18	0.039	−	−		53	2	0.71
	Positive	43	20	23		−	−		42	1	
Inflammatory cell infiltration	Negative	95	56	39	0.38	53	42	0.71	−	−	
	Positive	3	1	2		2	1		−	−	

*The presence of DM was defined as fasting blood glucose of 126 mg/dL or greater, HbA1c (NGSP) of 6.5% or greater, or treatment with antidiabetic drugs.
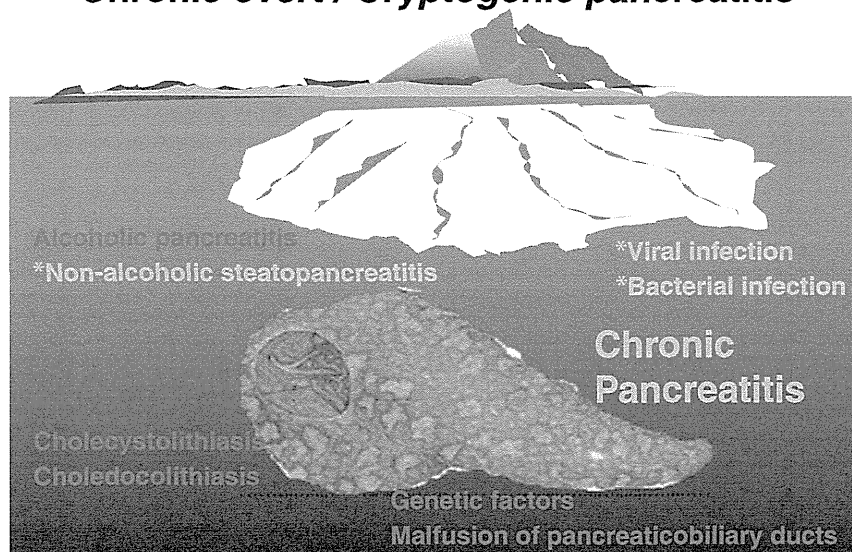†The Brinkman index is calculated as the number of cigarettes smoked per day multiplied by the number of years that the participant smoked.

TABLE 3. Comparison of Positivity Ratio of Pancreatic Fibrosis, Fatty Degeneration, and Inflammatory Cell Infiltration Between Patients With and Without Pancreatic Cancer

		Patients With Pancreatic Cancer	Patients Without Pancreatic Cancer	P
Fibrosis	Negative	11	57	<0.0001
	Positive	65	41	
Fatty degeneration	Negative	21	55	0.0002
	Positive	55	43	
Inflammatory cell infiltration	Negative	65	95	0.0061
	Positive	11	3	

PDAC-induced poor nutrition and cancer-associated complications led to weight loss in our PDAC patients. Because of this weight loss, PDAC patients demonstrated significantly lower BMI compared with non-PDAC controls in our study. These findings suggested that FPD is a risk factor for PDAC.

Next, we also performed univariate/multivariate analysis of each pancreatic histological change (secondary outcome) and clinical variables (age, sex, the presence of PDAC, Brinkman index, weekly ethanol intake, and BMI) (Supplementary Tables 2–4, http://links.lww.com/MPA/A303). Interestingly, the presence of PDAC was the only significant and independent determinant of each histological change. Other parameters were not statistically significant for any histological change.

DISCUSSION

The importance of chronic inflammation in the development of various cancers, especially those in digestive organs,

was recently recognized including *H. pylori*-associated gastric cancer, virus hepatitis, steatohepatitis-associated HCC, and chronic colitis-associated colon cancers.[30] In the chronic inflammatory condition, exogenous stimulants and intrinsic mediators of the inflammatory response, including proinflammatory cytokines, growth factors, and proteases such as tumor necrosis factor α, IL6, Hedgehog, and transforming growth factor β, may promote genetic and epigenetic changes that lead to oncogenesis.[30,31] We hypothesized that, like other cancers, PDAC also develops after chronic inflammation in the pancreas. To elucidate this issue, we investigated the background histology of the pancreas in PDAC patients and compared the histological changes in the pancreas with those in patients without PDAC.

We found significantly higher ratios of pancreatic fibrosis, fatty degeneration, and inflammatory cell infiltration in patients with PDAC compared with control patients. In addition, multivariate analysis showed that each pancreatic histological change was an
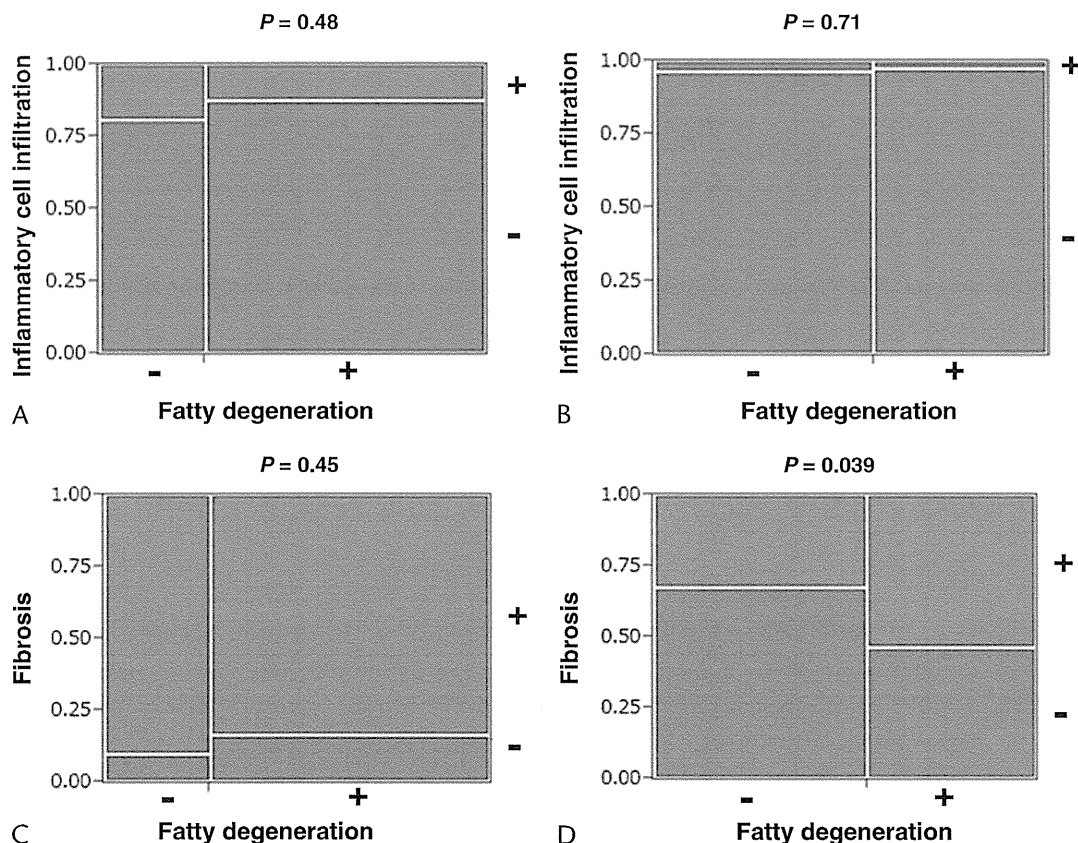
FIGURE 4. Correlation of fatty degeneration with lymphocyte infiltration and fibrosis. A and C represent the data from 76 patients with PDAC, and B and D represent the data from 98 non-PDAC patients. A correlation between fibrosis and fatty degeneration was observed in non-PDAC patients (P = 0.039), but not in patients with PDAC.

TABLE 4. Univariate and Multivariate Analysis Between PDAC and Clinical Variables

Factor	r	P	β	95% CI	P
Age	0.047	0.467			
Sex (female)	0.08	0.215			
Fatty degeneration (+)	0.25	0.0002	0.47	0.050–0.90	0.0298
Inflammatory cell infiltration (+)	0.18	0.0061	1.14	0.23–2.63	0.0413
Fibrosis (+)	0.39	<0.0001	0.88	0.43–1.37	0.0002
BMI	−0.31	0.0001	−0.25	−0.39 to 0.11	0.0006
Brinkman Index*	0.026	0.765			
Ethanol intake (g/w)	0.071	0.441			

*The Brinkman index is calculated as the number of cigarettes smoked per day multiplied by the number of years that the participant smoked. CI indicates confidence interval.

independent determinant of the presence of PDAC. These findings seem to be very similar to those of nonalcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH) followed by liver cirrhosis and HCC.[32,33] In the earlier stage of NASH/ASH, lymphocyte inflammation and steatosis are commonly observed. However, in the advanced stage, which is referred to as burnout NASH, less lymphocyte infiltration or fatty changes are observed despite the presence of advanced hepatic fibrosis. These pathological hepatic characteristics of burnout NASH are quite similar to the pancreatic characteristics of CP with PDAC observed in this study. Unlike liver disease, no reports exist of follow-up for CP with repeated biopsy. Therefore, we cannot conclude how many patients will develop CP or pancreatic fibrosis secondary to AFPD or NAFPD. In contrast, none of the patients in this study, with or without PDAC, had a history of clinical CP. Cryptogenic CP as a possible risk factor for PDAC was likely present in more people than expected (Fig. 5). Indeed, the rate of chronic pancreatic inflammation with fatty degeneration was significantly higher in patients with PDAC than in those without PDAC. These results

indicate that PDAC and CP may develop secondary to AFPD/ NAFPD. Patients with AFPD/NAFPD may be at risk for the future development of cryptogenic CP.

The most common genetic alteration in PDAC is the activating mutation of KRAS, which is an initial key event in pancreatic oncogenesis and is found in most cases of PDAC.[34] However, KRAS mutation itself is not sufficient for the development of PDAC; additional genetic alterations and signals from the tumor microenvironment are necessary for tumor progression.[31] Thus, the concept of PDAC has recently changed from a disease of malignant ductal cells alone to an integrated disease of cancer cells and surrounding stromal cells such as PSCs.[35–37] The PSCs are thought to be responsible for fibrotic changes in the pancreas.[38] Quiescent PSCs are located in the periacinar spaces and close to the basal aspect of acinar cells, capillaries, and terminal fibers.[38,39] The PSCs have the ability to secrete acetylcholine, and their role in the normal pancreas may be to form an interface between the nerve endings and acini.[40] Once the PSCs become active and obtain a myofibroblastlike phenotype

FIGURE 5. Hypothetical concept of CP as a precancerous lesion for pancreatic cancer. More patients than expected may have cryptogenic pancreatitis, which is caused by alcoholic and/or nonalcoholic fatty degeneration and viral/bacterial infection.

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in response to pancreatic injury or inflammation, returning to a quiescent state is difficult. Activated PSCs proliferate, migrate, and produce extracellular matrix components, cytokines, and chemokines.[41] In an experimental mouse model, *KRAS* overexpression alone in pancreatic acinar cells does not lead to carcinogenesis, but additional CP induction by repetitive cerulein injections induces metaplasia, dysplasia, and finally, cancer in situ.[42]

Our present study demonstrated that pancreatic fatty degeneration was closely associated with the presence of PDAC. Pancreatic fatty degeneration is closely associated with obesity, and the level of pancreatic fatty changes increases with the degree of obesity.[12,13] To elucidate whether pancreatic fatty degeneration was affected by clinical parameters in PDAC patients, we further performed multivariate analysis of pancreatic fatty degeneration using some lifestyle habit-associated predictors (age, BMI, DM, and weekly ethanol intake) and CRT. We found no parameters that significantly predicted pancreatic fatty degradation (Supplementary Table 5, http://links.lww.com/MPA/A303). Although these findings indicated that pancreatic fatty degradation in PDAC patients was not significantly correlated with lifestyle habits, we believe that PDAC-induced weight loss may have affected these results in our study.

In many people who undergo medical checkups, an echogenic pancreas, which indicates fatty change of the pancreas (AFPD or NAFPD), is frequently observed with ultrasonography. Because of increasing alcohol consumption[11] and obesity,[43] the number of patients with AFPD/NAFPD continues to increase.[12,44] An increased incidence of PDAC development in patients with CP was recently reported.[11,45,46] An increase in the number of patients with AFPD/NAFPD may result in an increase in the number of patients with PDAC. Recently, several elastography techniques using ultrasound or magnetic resonance imaging as noninvasive assessment methods for tissue fibrosis have been clinically used.[47–49] These techniques are useful for assessing pancreatic fibrosis. Identifying FPD patients as a high-risk group for PDAC using these examinations should lead to early diagnosis of PDAC. Although the major limitation of our present study was that our study was retrospective, a prospective study of whether cryptogenic CP (including FPD) could be a predicting factor for PDAC development could be performed using these noninvasive imaging tests.

In conclusion, our findings indicate that cryptogenic CP is an important predictive factor for PDAC. Further investigation is necessary to evaluate patients for subclinical CP in the healthy population and calculate the occurrence ratio of PDAC among them, which may enable the detection of PDAC at an early stage.

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Serum Fucosylated Haptoglobin as a Novel Prognostic Biomarker Predicting High-Gleason Prostate Cancer

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BACKGROUND. Fucosylation is an oligosaccharide modification associated with cancer and inflammation, which is catalyzed by fucosyltransferases. Fucosylated haptoglobin (Fuc-Hpt) has been identified as a novel biomarker for pancreatic cancer.
In this study, we evaluated serum Fuc-Hpt as a biomarker for prostate cancer, and investigated the expression of fucosyltransferases and haptoglobin in prostate cancer cell lines.
METHODS. We measured the preoperative serum Fuc-Hpt levels in 98 patients who underwent radical prostatectomy (RP) using an established lectin-antibody ELISA. Fucosyltransferase and haptoglobin mRNA and protein expressions in prostate cancer cell lines were determined using quantitative PCR and Western blotting.
RESULTS. Serum Fuc-Hpt levels were significantly associated with Gleason score (GS), but not prostate-specific antigen (PSA) levels. The area under the receiver-operator characteristics curve (AUC) for the prediction of GS ≥ 7 in prostatectomy specimens by Fuc-Hpt was 0.753, in contrast to the PSA AUC of 0.561 and the PSAD AUC of 0.558. The Fuc-Hpt AUC for the prediction of GS upgrade from GS 6 at biopsy to GS ≥ 7 after RP was 0.689, in contrast to the PSA AUC of 0.588 and PSAD AUC of 0.557. Multivariable analysis revealed that Fuc-Hpt levels were significantly associated with biochemical recurrence after prostatectomy. A high expression of alpha-(1–6) fucosyltransferase (*FUT8*) and haptoglobin was observed in prostate cancer cell line, suggesting that certain kinds of prostate cancer cells produce Fuc-Hpt.
CONCLUSION. Elevated serum Fuc-Hpt level could be a novel cancer biomarker for predicting the prognosis of patients with prostate cancer, particularly those with high GSs. *Prostate 74:1052–1058, 2014.* © 2014 Wiley Periodicals, Inc.

KEY WORDS: haptoglobin; prostate cancer; fucosylation; tumor marker; fucosyltransferase

INTRODUCTION

Although most prostate cancer patients are diagnosed with clinically localized disease, approximately 30–40% of patients who receive definitive therapy experience biochemical recurrence. Active surveillance is also offered to patients with low-risk prostate cancer; however, 14–16% of these patients experienced disease progression and later require definitive therapy [1,2]. Of patients diagnosed with low-risk prostate cancer using biopsy, 21% had an adverse pathological finding resulting in a Gleason score (GS) upgrade, and 24% had non-organ confined disease at radical prosta-

tectomy (RP) [3]. Prostate-specific antigen (PSA) levels and GSs obtained from biopsy specimens are currently used as indicators to guide the prognosis and treatment of prostate cancer. However, a more precise indicator of disease severity is critical to the selection

of an optimal treatment modality for localized prostate cancer.

Fucosylation is an important oligosaccharide modification associated with cancer and inflammation. Certain fucosylated glycoproteins catalyzed by alpha-(1–3) (*FUTs 3–7* and *FUTs 9–11*)/alpha-(1–6) (*FUT8*) fucosyltransferases have been identified as cancer biomarkers, such as pancreatic cancer and colon cancer [4]. Alpha-(1–3)-fucosyltransferase is involved in the synthesis of Lewis-type fucosylation, and alpha-(1–6) fucosylation is involved in core fucosylation, whereas alpha-(1–2)-fucosyltransferase (*FUT1* and *FUT2*) is involved in H-type fucosylation. We have previously demonstrated the use of fucosylated haptoglobin (Fuc-Hpt) as a novel biomarker for patients with pancreatic cancer and developed a lectin-antibody enzyme-linked immunosorbent assay (ELISA) to measure the serum Fuc-Hpt levels [5,6]. Haptoglobin is produced mainly by the liver and exhibits low levels of glycan fucosylation in healthy controls [4]. In the case of pancreatic cancer, we expected the origin of Fuc-Hpt to be metastatic lesions in the liver, because high expression of haptoglobin and *FUT8* was not observed. As metastatic cancers in the liver induce the production of Fuc-Hpt, serum Fuc-Hpt levels were also found to be associated with prognosis after colorectal cancer surgery [7]. Elevated serum Fuc-Hpt levels have been reported in prostate cancer patients compared with levels in normal controls, and the N-glycan structure attached to its beta-chain has been analyzed in detail [8]. However, there have been no reports on the relationship between serum Fuc-Hpt levels and prostate cancer prognosis.

In the present study, we evaluated serum Fuc-Hpt levels as a preoperative prognostic biomarker in localized prostate cancer patients who underwent RP. Furthermore, we investigated the expression of *FUT8* and haptoglobin in several different cell lines.

MATERIALS AND METHODS

Serum Samples

Ninety-eight previously collected serum samples were obtained from our biorepository and linked to information about the patients' prostate health status as well as relevant demographic and pathologic data. These serum samples were collected preoperatively from patients who underwent RP at Osaka University Hospital. Serum samples were stored at −80°C until analysis. The serum PSA levels measured before biopsy were obtained from patient medical records. We excluded serum samples from patients with positive surgical margins. Ethical approval was obtained

from our Institutional Review Board and all patients provided written informed consent.

Lectin-Antibody ELISA for Fuc-Hpt

Lectin-antibody ELISA for Fuc-Hpt was performed as previously described [7]. Concisely, the Fab fragment of anti-human haptoglobin IgG (Dako, Carpinteria, CA) was coated on the bottom of a 96-well ELISA plate. Coated plates were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin for 1 hr, followed by washing with PB containing 0.1% Tween 20 (PBS-T). A 50-μL aliquot of sera was placed in each well and incubated for 1 hr at room temperature. To detect Fuc-Hpt, 1/1,000 diluted biotinylated aleuria aurantica lectin was placed in each well and incubated at room temperature for 1 hr. Peroxidase-conjugated avidin was added to each well and incubated at room temperature for 1 hr. Tetramethylbenzidine was added to each well, followed by 15-min incubation for development. To stop development, 1 N sulfuric acid was added to each well. A standard curve for Fuc-Hpt was obtained using the standard Fuc-Hpt purchased from Takara Bio Inc. (Shiga, Japan).

Statistical Analysis

Fuc-Hpt levels were compared between groups using the Mann–Whitney U test or Kruskal–Wallis test. A Kaplan–Meier curve and log–rank test were used to explore the association between the parameters and patient survival. Univariable and multivariable analyses were performed using a Cox proportional hazards model to predict biochemical recurrence-free survival (bRFS). Variables entered into the model for bRFS analysis included patient age, initial PSA, Fuc-Hpt level, GS, and pathological finding of capsular and seminal vesicle invasion. Biochemical recurrence after RP was defined as a sustained elevation of serum PSA level at >0.2 ng/ml on two or more occasions, and the date of recurrence was recorded as the first date the PSA value was measured as >0.2 ng/ml. All probability (P) values were two-sided, with statistical significance set at $P < 0.05$. All statistical analyses were performed using SPSS version 11.0.1 (SPSS, Chicago, IL) and GraphPadPrizm 5 (Graph-Pad Software, La Jolla, CA).

Cell Culture

Cell lines, LNCaP, 22Rv1, DU145, and PC3 were purchased from Riken Cell Banks (Saitama, Japan). The cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum

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and 1% penicillin/streptomycin at 37°C containing 5% CO_2.

All cell lines in subconfluent conditions were used for Western blot and quantitative polymerase chain reaction (qPCR) experiments.

Quantitative PCR

Total RNA was extracted from LNCaP, 22Rv1, PC3, and DU145. Total RNA (1 μg) was treated with DNase I (1U, Invitrogen, Carlsbad, CA) for 15 min.

cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to manufacturer's instructions. Amplification of specific PCR products was performed using the THUNDER-BIRD SYBR qPCR mix (TOYOBO). The following primers (each 300 nM) were used in 20-μL reactions: FUT1, 5'-AGACTTTGCCCTGCTCACAC-3' (forward) and 5'-TGAAGTTGGCCAGGTAGACAG-3' (reverse); FUT2, 5'-CAGATGCCTTTCTCCTTTCC-3' (forward) and 5'-ACTCCCACATGGCTTGAATC-3' (reverse); FUT3, 5'-CTGGATCTGGTTCAACTTGG-3' (forward) and 5'-CGGTAGGACATGGTGAGATTG-3' (reverse); FUT4, 5'-GGGTTTGGATGAACTTCGAG-3' (forward) and 5'-AGCCATAAGGCACAAAGACG-3' (reverse); FUT5, 5'-CTTATGGCAGTGGAACCTGTC-3' (forward) and 5'-AGCCACGGGTGTGTTAAAAG-3' (reverse); FUT6, 5'-TCCTATCTGCGTGTGTCTCAAG-3' (forward) and 5'-TGTTAAAAGGCCACGTCCAC-3' (reverse); FUT7, 5'-GTGCATGTGGATGACTTTGG-3' (forward) and 5'-GGCAAAGAAGCGTTGGTATC-3' (reverse); FUT8, 5'-ATCCTGATGCCTCTGCAAAC-3' (forward) and 5'-GGGTTGGTGAGCATAAATGG-3' (reverse); FUT9, 5'-CCCAAAAGAGTGGCATTGAG-3' (forward) and 5'-CGAAGGGATTTGTGCTTACC-3' (reverse); ribosomal protein L4 (RPL4), 5'-GACTTAA-CACACGAGGAGATGC-3' (forward) and 5'-GCATG-CTGTGCACATTTAGG-3' (reverse). Cycle parameters were as follows: denaturation at 95°C for 3 min, and 40 cycles of denaturation at 95°C for 15 sec, annealing at 59°C for 10 sec, and polymerization at 72°C for 25 sec. Reverse transcriptase-qPCR was performed using an Mx3000P Real-Time qPCR System (Agilent). Expression levels of the genes of interest were normalized to RPL4 and calculated based on the $2^{-\Delta\Delta CT}$ method. The results were expressed as relative ratios to RPL4.

Western Blot Analysis

After washing with ice-cold PBS, cells were harvested in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with Halt protease inhibitor cocktail (Thermo Scientific). Total cellular protein concentrations were determined using a bicinchoninic acid protein assay reagent (Thermo Scientific).

The lysates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (15-μg protein per lane) under reducing conditions, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with mouse monoclonal anti-human FUT8 antibodies at a dilution of 1:1,000, mouse monoclonal anti-human haptoglobin antibodies at a dilution of 1:2,000, or mouse monoclonal anti-human beta-actin antibodies at a dilution of 1:1,000, followed by horseradish peroxidase-conjugated secondary antibodies, and developed with the Super Signal West Dura Extended Duration Substrate kit (Thermo Scientific).
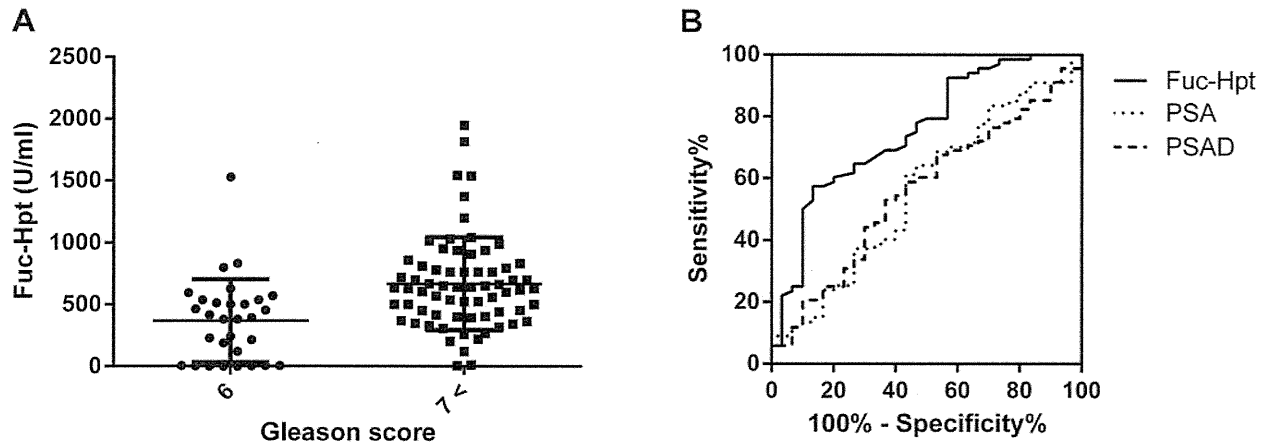
RESULTS

Association of Fuc-Hpt Levels With Gleason Scores

The clinic-pathological characteristics of patients are shown in Table I. There were no correlations between Fuc-Hpt levels and PSA (Spearman $r = -0.02$, $P = 0.81$) or age (Spearman's $r = 0.14$, $P = 0.14$). Preoperative serum Fuc-Hpt levels were associated with GS; Fuc-Hpt levels were significantly higher in patients with GS ≥ 7 (median: 631 U/ml; range: 4.8–1,947; U/ml; $P < 0.001$) than in those with GS 6 (median: 385 U/ml; range: 1.4–1,529; $P < 0.001$; Fig. 1A). Stratification of patients by highest Gleason pattern, including tertiary pattern, showed a median Fuc-Hpt level of 379 U/ml (range: 1.4–1,529) in patients with the highest Gleason pattern 3, 637 U/ml (range: 119–1,947) in patients with highest Gleason pattern 4, and 512 U/ml (range: 4.8–1,542) in patients with the highest Gleason pattern 5 ($P < 0.0001$). The area under the receiver-operator characteristic (ROC) curve (AUC) for the prediction of GS ≥ 7 in prostatectomy specimens by Fuc-Hpt was

TABLE I. Patient Demographics

Age (median [range])	65 (47–76)
PSA (median [range])	6.92 (2.60–72.2)
Gleason score at Biopsy, n (%)	
6	38 (38.8)
7	39 (39.8)
8	12 (12.2)
9	9 (9.2)
Gleason score at RP, n (%)	
6	29 (29.6)
7	50 (51.0)
8	3 (3.1)
9	16 (16.3)
Capsular invasion, n (%)	25 (25.5)
Seminal vesicle invasion, n (%)	7 (7.1)

Fig. 1. Serum fucosylated haptoglobin (Fuc-Hpt) levels are associated with the Gleason score (GS) of radical prostatectomy (RP) specimens. **(A)** Serum Fuc-Hpt levels in prostate cancer patients who underwent RP. **(B)** The area under the receiver-operator characteristics curve for Fuc-Hpt of the prediction of GS $\geq$7 in prostatectomy specimens (Fuc-Hpt, solid curve; PSA, dotted curve; PSA density, dashed curve).

0.753 (95% confidence interval [CI], 0.648–0.858), in contrast to the PSA AUC of 0.561 (95% CI, 0.435–0.686) and the prostate-specific antigen density (PSAD) AUC of 0.558 (95% CI, 0.436–0.679; Fig. 1B). At the optimal cutoff, sensitivity was 57.4% and specificity was 86.7%. Of 38 patients with GS 6 at biopsy, 15 had GS upgrade to GS $\geq$7 after prostatectomy. Fuc-Hpt levels were higher in patients with GS upgrade to $\geq$7 (median: 618 U/L; range: 119–1,015) than in those without GS upgrade (median: 452 U/L; range: 1.4–1,529; $P = 0.0504$). Of 60 patients with GS $\geq$7 at biopsy, 7 had GS downgrade to GS 6 after prostatectomy. Fuc-Hpt levels were significantly lower in these patients (median: 188; range: 1.6–500) than in those with the same GS of 7–9 (median: 640; range: 4.8–1,947; $P = 0.0001$). The Fuc-Hpt AUC for the prediction of GS upgrade from GS 6 after biopsy to GS $\geq$7 after RP was 0.689 (95% CI: 0.517–0.863), in contrast to the PSA AUC of 0.588 (95% CI: 0.395–0.781) and the PSAD AUC of 0.557 (95% CI: 0.351–

0.762). At the optimal cutoff for the prediction of GS upgrade, the sensitivity was 60.0% and the specificity was 82.6%.

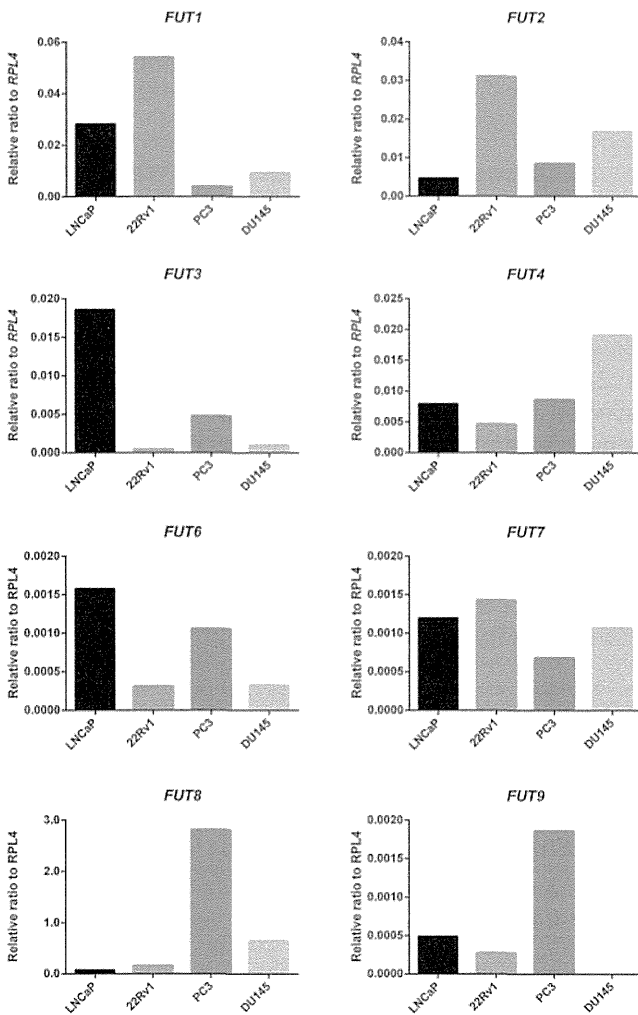### Association of Fuc-Hpt Levels With Biochemical Recurrence

We analyzed the association of preoperative serum Fuc-Hpt levels with clinico-biochemical PSA recurrence. The median follow-up period was 44 months (ranged 9–118 months). PSA recurrence was observed in 24 of 98 patients (24.4%). Univariable analysis revealed that GS (8–9 vs. 6) and Fuc-Hpt levels were significantly associated with PSA recurrence ($P = 0.011$ and $P = 0.037$, respectively). Multivariable analysis with GS, Fuc-Hpt levels, PSA levels, age, and the pathological finding of capsular invasion and seminal vesicle involvement revealed that only Fuc-Hpt levels was significantly associated with PSA recurrence ($P = 0.024$) (Table II).

TABLE II. Cox Proportional Hazards Analysis to Predict a Patient's Biochemical Recurrence-Free Survival
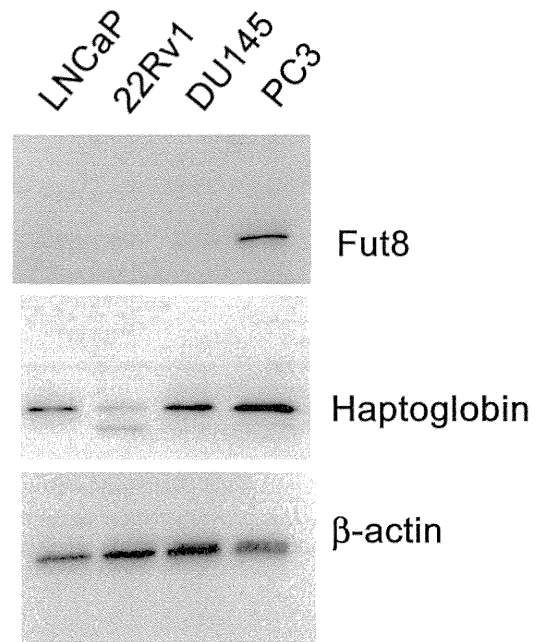
	Univariate		Mutivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Age, year	1.06 (0.98–1.14)	0.10		
PSA (ng/dl) (continuous)	1.03 (0.98–1.09)	0.19		
Gleason score (vs. 6)				
7	2.38 (0.66–8.54)	0.18		
8–9	5.84 (1.49–22.9)	0.011		
Capsular invasion	1.22 (0.47–3.17)	0.67		
Seminal vesicle invasion	2.93 (0.85–10.04)	0.087		
Fuc-Hpt ($\times$1,000 U/ml, continuous)	2.65 (1.05–6.66)	0.037	2.905 (1.14–7.34)	0.024

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Expression of FUT8 mRNA and Proteins in Prostate Cancer Cell Lines

The origin of elevated serum Fuc-Hpt levels in cancer patients warrant further discussion. It is believed that cancer cells, their surrounding tissues, and infiltrating lymphocytes are all potential candidates for Fuc-Hpt-producing cells. To determine whether haptoglobin and FUT8 are expressed in prostate cancer cell lines, we first analyzed the mRNA expressions of several kinds of fucosyltransferases by qPCR. As shown in Figure 2, PC3 strongly expressed FUT8 mRNA, DU145 moderately expressed FUT8 mRNA, and LNCaP and 22Rv1 weakly expressed FUT8 mRNA. The mRNA expressions levels of other fucosyltransferases (FUT1, FUT2, FUT3, FUT4, FUT6, FUT7, and FUT9) were lower in prostate cancer cell lines. Western blots of lysates of LNCaP, 22Rv1, and DU145 revealed weak signals detected by monoclonal

Fig. 3. FUT8 and haptoglobin expression by Western blotting in four prostate cancer cell lines. Total cell lysates from prostate cancer cell lines are immunoblotted using antibodies for FUT8 (upper panel), haptoglobin (middle panel), and beta-actin (lower panel).

antibody against human FUT8, and a robust signal in lysates of PC3 (Fig. 3). Robust signals were detected in all four cell lines by monoclonal antibody against human haptoglobin. FUT8 catalyzes alpha-(1–6)-fucosylation, which contributes to core-type fucosylation. Thus, prostate cancer cells could be a potential source of Fuc-Hpt in patients with prostate cancer.

Fig. 2. Expression of fucosyltransferases (FUT1, FUT2, FUT3, FUT4, FUT6, FUT7, FUT8, and FUT9) in prostate cancer cell lines by quantitative polymerase chain reaction.

DISCUSSION

Almost 50% of patients were diagnosed with low-risk prostate cancer in a PSA screening cohort, which would not be life threatening [9]. The overall survival of patients treated with active surveillance for low-risk prostate cancer is comparable to that of patients who undergo prostatectomy. However, approximately 14–16% of the PSA-positive patients undergoing active surveillance experienced disease progression and required definitive therapy [1,2]. Half of the patients with localized prostate cancer had adverse pathology at RP [10,11]. Therefore, the development of biomarkers to detect high-risk prostate cancer and predict prognosis is essential to avoid overtreatment of a disease that is not life threatening or underestimation of aggressive disease. In the present study, we demonstrated that preoperative Fuc-Hpt levels were associated with high GS in RP specimens. PSA and PSAD are reported to be significant predictors of GS upgrade in men diagnosed with low-risk disease [10].

We also demonstrated that serum Fuc-Hpt could predict GS upgrade better than PSA and PSAD, although our cohort size was small. The Fuc-Hpt test may prove useful for the prediction of GS upgrade, particularly in low-risk prostate cancer patients who can then undergo less invasive treatment such as active surveillance.

The origin of serum Fuc-Hpt in patients with prostate cancer is still not fully understood, similar to that in other types of cancer. While most haptoglobin is produced by hepatocytes, ectopic expression of haptoglobin has been observed during infection, inflammation, and cancer [4]. Fucosylation of the haptoglobin beta-chain is catalyzed by alpha-(1–3)/alpha-(1–6)-fucosyltransferases. Fucosyltransferases globally modify surface antigens, receptors, adhesion molecules, as well as haptoglobin. They are involved in the regulation of genes associated with malignancy, such as epithelial–mesenchymal transition [12]. Serum Fuc-Hpt level is increased in patients with advanced cancers, including pancreatic cancer, ovarian cancer, lung cancer, and hepatocellular carcinoma [5,13,14]. Serum Fuc-Hpt in colon cancer patients could originate from lymphocytes or hepatocytes around metastatic cancer cells in the liver [7]. Fuc-Hpt production is induced in hepatocytes and hepatoma cells treated with interleukin (IL) 6 [5]. It is hypothesized that hepatocytes around metastatic pancreatic cancer in the liver produce Fuc-Hpt through IL-6 and loss of cellular polarity in metastatic lesions. In prostate cancer, metastasis to the liver is uncommon compared with that to the lymph nodes or bones [15]. However, elevated IL-6 levels were observed in the sera of prostate cancer patients, particularly at advanced stages, with bone metastasis. The majority of the fucosylation was reported as Lewis-type in prostate cancer [8]. We found that prostate cancer cell lines expressed both *FUT8* and haptoglobin, but considerably lower levels of alpha-(1–3)-fucosyltransferase mRNA, and it is suggested that prostate cancer cells mainly secrete core Fuc-Hpt. Lewis-type Fuc-Hpt may be produced by other cells around the prostate cancer.

Expression of *FUT8* is high in several kinds of cancer cells and white blood cells, and very low in normal hepatocytes [4]. A high expression of *FUT8* is observed in non-small cell lung cancer (NSCLC) patients and is associated with poor survival due to upregulation of growth factor-receptor signaling [12]. Increases in *FUT8* expression and core fucosylation of cell surface receptors might promote cell proliferation in high GS prostate cancer. Dysregulation of *FUT8*, with for example, siRNA and/or a neutral antibody for core fucose, might be a possible target for aggressive prostate cancer.

We demonstrated the association of Fuc-Hpt levels with GSs in serum samples obtained before prostatectomy, but not before biopsy. The Fuc-Hpt test may also be useful in a screening setting when combined with the PSA test. Fujimura et al. reported that haptoglobin levels in the sera of prostate cancer patients were significantly higher than those in the sera of patients with benign prostate disease and healthy subjects [8]. The serum samples obtained before biopsy should also be tested to detect intermediate and high-risk prostate cancer. This was a relatively small retrospective cohort study, with a follow-up period of 44 months. Larger scale studies are warranted to confirm these findings.

In conclusion, serum Fuc-Hpt levels were associated with GS, and could be used as a prognostic marker of biochemical recurrence in patients with localized prostate cancer who undergo RP.

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Serum Fucosylated Haptoglobin as a Novel Prognostic Biomarker Predicting High-Gleason Prostate Cancer

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BACKGROUND. Fucosylation is an oligosaccharide modification associated with cancer and inflammation, which is catalyzed by fucosyltransferases. Fucosylated haptoglobin (Fuc-Hpt) has been identified as a novel biomarker for pancreatic cancer.
In this study, we evaluated serum Fuc-Hpt as a biomarker for prostate cancer, and investigated the expression of fucosyltransferases and haptoglobin in prostate cancer cell lines.
METHODS. We measured the preoperative serum Fuc-Hpt levels in 98 patients who underwent radical prostatectomy (RP) using an established lectin-antibody ELISA. Fucosyl-transferase and haptoglobin mRNA and protein expressions in prostate cancer cell lines were determined using quantitative PCR and Western blotting.
RESULTS. Serum Fuc-Hpt levels were significantly associated with Gleason score (GS), but not prostate-specific antigen (PSA) levels. The area under the receiver-operator characteristics curve (AUC) for the prediction of GS ≥ 7 in prostatectomy specimens by Fuc-Hpt was 0.753, in contrast to the PSA AUC of 0.561 and the PSAD AUC of 0.558. The Fuc-Hpt AUC for the prediction of GS upgrade from GS 6 at biopsy to GS ≥ 7 after RP was 0.689, in contrast to the PSA AUC of 0.588 and PSAD AUC of 0.557. Multivariable analysis revealed that Fuc-Hpt levels were significantly associated with biochemical recurrence after prostatectomy. A high expression of alpha-(1–6) fucosyltransferase (*FUT8*) and haptoglobin was observed in prostate cancer cell line, suggesting that certain kinds of prostate cancer cells produce Fuc-Hpt.
CONCLUSION. Elevated serum Fuc-Hpt level could be a novel cancer biomarker for predicting the prognosis of patients with prostate cancer, particularly those with high GSs.
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KEY WORDS: haptoglobin; prostate cancer; fucosylation; tumor marker; fucosyltransferase

INTRODUCTION

Although most prostate cancer patients are diagnosed with clinically localized disease, approximately 30–40% of patients who receive definitive therapy experience biochemical recurrence. Active surveillance is also offered to patients with low-risk prostate cancer; however, 14–16% of these patients experienced disease progression and later require definitive therapy [1,2]. Of patients diagnosed with low-risk prostate cancer using biopsy, 21% had an adverse pathological finding resulting in a Gleason score (GS) upgrade, and 24% had non-organ confined disease at radical prosta-

tectomy (RP) [3]. Prostate-specific antigen (PSA) levels and GSs obtained from biopsy specimens are currently used as indicators to guide the prognosis and treatment of prostate cancer. However, a more precise indicator of disease severity is critical to the selection

of an optimal treatment modality for localized prostate cancer.

Fucosylation is an important oligosaccharide modification associated with cancer and inflammation. Certain fucosylated glycoproteins catalyzed by alpha-(1–3) (FUTs 3–7 and FUTs 9–11)/alpha-(1–6) (FUT8) fucosyltransferases have been identified as cancer biomarkers, such as pancreatic cancer and colon cancer [4]. Alpha-(1–3)-fucosyltransferase is involved in the synthesis of Lewis-type fucosylation, and alpha-(1–6) fucosylation is involved in core fucosylation, whereas alpha-(1–2)-fucosyltransferase (FUT1 and FUT2) is involved in H-type fucosylation. We have previously demonstrated the use of fucosylated haptoglobin (Fuc-Hpt) as a novel biomarker for patients with pancreatic cancer and developed a lectin-antibody enzyme-linked immunosorbent assay (ELISA) to measure the serum Fuc-Hpt levels [5,6]. Haptoglobin is produced mainly by the liver and exhibits low levels of glycan fucosylation in healthy controls [4]. In the case of pancreatic cancer, we expected the origin of Fuc-Hpt to be metastatic lesions in the liver, because high expression of haptoglobin and FUT8 was not observed. As metastatic cancers in the liver induce the production of Fuc-Hpt, serum Fuc-Hpt levels were also found to be associated with prognosis after colorectal cancer surgery [7]. Elevated serum Fuc-Hpt levels have been reported in prostate cancer patients compared with levels in normal controls, and the N-glycan structure attached to its beta-chain has been analyzed in detail [8]. However, there have been no reports on the relationship between serum Fuc-Hpt levels and prostate cancer prognosis.

In the present study, we evaluated serum Fuc-Hpt levels as a preoperative prognostic biomarker in localized prostate cancer patients who underwent RP. Furthermore, we investigated the expression of FUT8 and haptoglobin in several different cell lines.

MATERIALS AND METHODS

Serum Samples

Ninety-eight previously collected serum samples were obtained from our biorepository and linked to information about the patients' prostate health status as well as relevant demographic and pathologic data. These serum samples were collected preoperatively from patients who underwent RP at Osaka University Hospital. Serum samples were stored at −80°C until analysis. The serum PSA levels measured before biopsy were obtained from patient medical records. We excluded serum samples from patients with positive surgical margins. Ethical approval was obtained

from our Institutional Review Board and all patients provided written informed consent.

Lectin-Antibody ELISA for Fuc-Hpt

Lectin-antibody ELISA for Fuc-Hpt was performed as previously described [7]. Concisely, the Fab fragment of anti-human haptoglobin IgG (Dako, Carpinteria, CA) was coated on the bottom of a 96-well ELISA plate. Coated plates were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin for 1 hr, followed by washing with PB containing 0.1% Tween 20 (PBS-T). A 50-μL aliquot of sera was placed in each well and incubated for 1 hr at room temperature. To detect Fuc-Hpt, 1/1,000 diluted biotinylated aleuria aurantica lectin was placed in each well and incubated at room temperature for 1 hr. Peroxidase-conjugated avidin was added to each well and incubated at room temperature for 1 hr. Tetramethylbenzidine was added to each well, followed by 15-min incubation for development. To stop development, 1 N sulfuric acid was added to each well. A standard curve for Fuc-Hpt was obtained using the standard Fuc-Hpt purchased from Takara Bio Inc. (Shiga, Japan).

Statistical Analysis

Fuc-Hpt levels were compared between groups using the Mann–Whitney U test or Kruskal–Wallis test. A Kaplan–Meier curve and log–rank test were used to explore the association between the parameters and patient survival. Univariable and multivariable analyses were performed using a Cox proportional hazards model to predict biochemical recurrence-free survival (bRFS). Variables entered into the model for bRFS analysis included patient age, initial PSA, Fuc-Hpt level, GS, and pathological finding of capsular and seminal vesicle invasion. Biochemical recurrence after RP was defined as a sustained elevation of serum PSA level at >0.2 ng/ml on two or more occasions, and the date of recurrence was recorded as the first date the PSA value was measured as >0.2 ng/ml. All probability (P) values were two-sided, with statistical significance set at $P < 0.05$. All statistical analyses were performed using SPSS version 11.0.1 (SPSS, Chicago, IL) and GraphPadPrizm 5 (Graph-Pad Software, La Jolla, CA).

Cell Culture

Cell lines, LNCaP, 22Rv1, DU145, and PC3 were purchased from Riken Cell Banks (Saitama, Japan). The cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum

and 1% penicillin/streptomycin at 37°C containing 5% CO_2.

All cell lines in subconfluent conditions were used for Western blot and quantitative polymerase chain reaction (qPCR) experiments.

Quantitative PCR

Total RNA was extracted from LNCaP, 22Rv1, PC3, and DU145. Total RNA (1 μg) was treated with DNase I (1U, Invitrogen, Carlsbad, CA) for 15 min.

cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to manufacturer's instructions. Amplification of specific PCR products was performed using the THUNDER-BIRD SYBR qPCR mix (TOYOBO). The following primers (each 300 nM) were used in 20-μL reactions: FUT1, 5'-AGACTTTGCCCTGCTCACAC-3' (forward) and 5'-TGAAGTTGGCCAGGTAGACAG-3' (reverse); FUT2, 5'-CAGATGCCTTTCTCCTTTCC-3' (forward) and 5'-ACTCCCACATGGCTTGAATC-3' (reverse); FUT3, 5'-CTGGATCTGGTTCAACTTGG-3' (forward) and 5'-CGGTAGGACATGGTGAGATTG-3' (reverse); FUT4, 5'-GGGTTTGGATGAACTTCGAG-3' (forward) and 5'-AGCCATAAGGCACAAAGACG-3' (reverse); FUT5, 5'-CTTATGGCAGTGGAACCTGTC-3' (forward) and 5'-AGCCACGGGTGTGTTAAAAG-3' (reverse); FUT6, 5'-TCCTATCTGCGTGTGTCTCAAG-3' (forward) and 5'-TGTTAAAAGGCCACGTCCAC-3' (reverse); FUT7, 5'-GTGCATGTGGATGACTTTGG-3' (forward) and 5'-GGCAAAGAAGCGTTGGTATC-3' (reverse); FUT8, 5'-ATCCTGATGCCTCTGCAAAC-3' (forward) and 5'-GGGTTGGTGAGCATAAATGG-3' (reverse); FUT9, 5'-CCCAAAAGAGTGGCATTGAG-3' (forward) and 5'-CGAAGGGATTTGTGCTTACC-3' (reverse); ribosomal protein L4 (RPL4), 5'-GACTTAA-CACACGAGGAGATGC-3' (forward) and 5'-GCATG-CTGTGCACATTTAGG-3' (reverse). Cycle parameters were as follows: denaturation at 95°C for 3 min, and 40 cycles of denaturation at 95°C for 15 sec, annealing at 59°C for 10 sec, and polymerization at 72°C for 25 sec. Reverse transcriptase-qPCR was performed using an Mx3000P Real-Time qPCR System (Agilent). Expression levels of the genes of interest were normalized to RPL4 and calculated based on the $2^{-\Delta\Delta CT}$ method. The results were expressed as relative ratios to RPL4.

Western Blot Analysis

After washing with ice-cold PBS, cells were harvested in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with Halt protease inhibitor cocktail (Thermo Scientific). Total cellular protein concentrations were determined using a bicinchoninic acid protein assay reagent (Thermo Scientific).

The lysates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (15-μg protein per lane) under reducing conditions, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with mouse monoclonal anti-human FUT8 antibodies at a dilution of 1:1,000, mouse monoclonal anti-human haptoglobin antibodies at a dilution of 1:2,000, or mouse monoclonal anti-human beta-actin antibodies at a dilution of 1:1,000, followed by horseradish peroxidase-conjugated secondary antibodies, and developed with the Super Signal West Dura Extended Duration Substrate kit (Thermo Scientific).
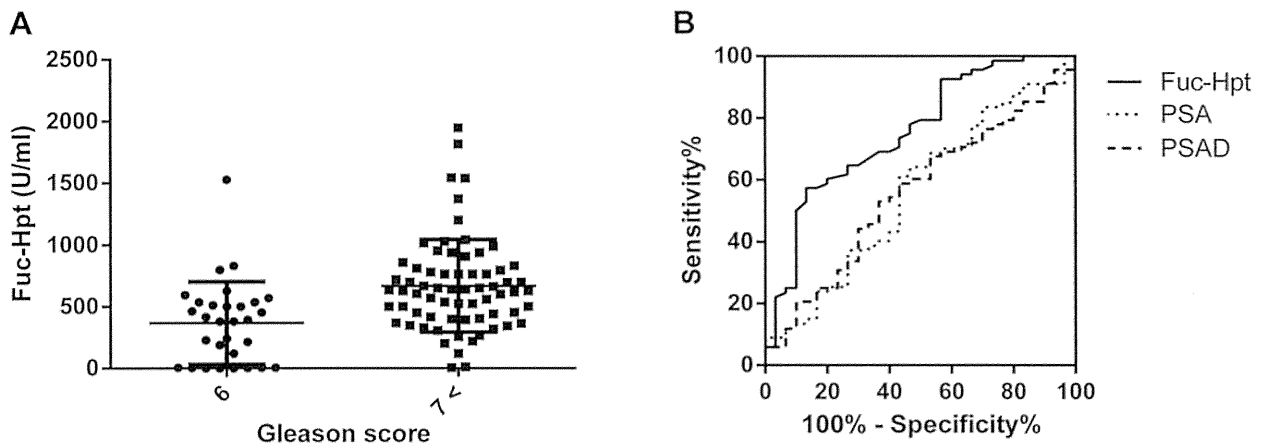
RESULTS

Association of Fuc-Hpt Levels With Gleason Scores

The clinic-pathological characteristics of patients are shown in Table I. There were no correlations between Fuc-Hpt levels and PSA (Spearman $r = -0.02$, $P = 0.81$) or age (Spearman's $r = 0.14$, $P = 0.14$). Preoperative serum Fuc-Hpt levels were associated with GS; Fuc-Hpt levels were significantly higher in patients with GS ≥ 7 (median: 631 U/ml; range: 4.8–1,947; U/ml; $P < 0.001$) than in those with GS 6 (median: 385 U/ml; range: 1.4–1,529; $P < 0.001$; Fig. 1A). Stratification of patients by highest Gleason pattern, including tertiary pattern, showed a median Fuc-Hpt level of 379 U/ml (range: 1.4–1,529) in patients with the highest Gleason pattern 3, 637 U/ml (range: 119–1,947) in patients with highest Gleason pattern 4, and 512 U/ml (range: 4.8–1,542) in patients with the highest Gleason pattern 5 ($P < 0.0001$). The area under the receiver-operator characteristic (ROC) curve (AUC) for the prediction of GS ≥ 7 in prostatectomy specimens by Fuc-Hpt was

TABLE I. Patient Demographics

Age (median [range])	65 (47–76)
PSA (median [range])	6.92 (2.60–72.2)
Gleason score at Biopsy, n (%)	
6	38 (38.8)
7	39 (39.8)
8	12 (12.2)
9	9 (9.2)
Gleason score at RP, n (%)	
6	29 (29.6)
7	50 (51.0)
8	3 (3.1)
9	16 (16.3)
Capsular invasion, n (%)	25 (25.5)
Seminal vesicle invasion, n (%)	7 (7.1)

A

B

Fig. 1. Serum fucosylated haptoglobin (Fuc-Hpt) levels are associated with the Gleason score (GS) of radical prostatectomy (RP) specimens. (**A**) Serum Fuc-Hpt levels in prostate cancer patients who underwent RP. (**B**) The area under the receiver-operator characteristics curve for Fuc-Hpt of the prediction of GS ≥ 7 in prostatectomy specimens (Fuc-Hpt, solid curve; PSA, dotted curve; PSA density, dashed curve).

0.753 (95% confidence interval [CI], 0.648–0.858), in contrast to the PSA AUC of 0.561 (95% CI, 0.435–0.686) and the prostate-specific antigen density (PSAD) AUC of 0.558 (95% CI, 0.436–0.679; Fig. 1B). At the optimal cutoff, sensitivity was 57.4% and specificity was 86.7%. Of 38 patients with GS 6 at biopsy, 15 had GS upgrade to GS ≥ 7 after prostatectomy. Fuc-Hpt levels were higher in patients with GS upgrade to ≥ 7 (median: 618 U/L; range: 119–1,015) than in those without GS upgrade (median: 452 U/L; range: 1.4–1,529; $P = 0.0504$). Of 60 patients with GS ≥ 7 at biopsy, 7 had GS downgrade to GS 6 after prostatectomy. Fuc-Hpt levels were significantly lower in these patients (median: 188; range: 1.6–500) than in those with the same GS of 7–9 (median: 640; range: 4.8–1,947; $P = 0.0001$). The Fuc-Hpt AUC for the prediction of GS upgrade from GS 6 after biopsy to GS ≥ 7 after RP was 0.689 (95% CI: 0.517–0.863), in contrast to the PSA AUC of 0.588 (95% CI: 0.395–0.781) and the PSAD AUC of 0.557 (95% CI: 0.351–

0.762). At the optimal cutoff for the prediction of GS upgrade, the sensitivity was 60.0% and the specificity was 82.6%.

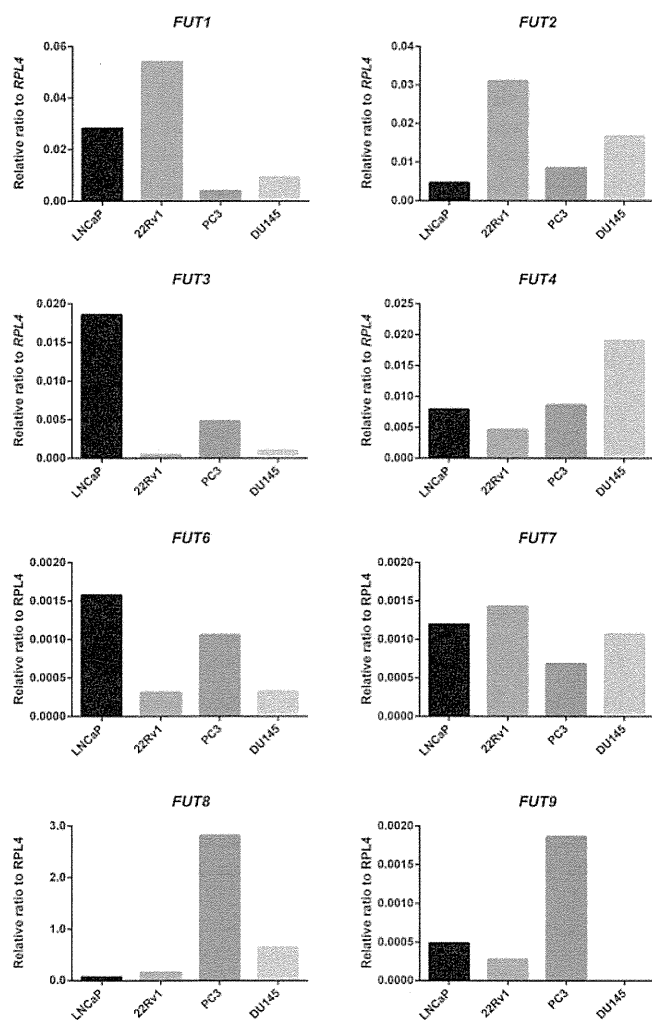### Association of Fuc-Hpt Levels With Biochemical Recurrence

We analyzed the association of preoperative serum Fuc-Hpt levels with clinico-biochemical PSA recurrence. The median follow-up period was 44 months (ranged 9–118 months). PSA recurrence was observed in 24 of 98 patients (24.4%). Univariable analysis revealed that GS (8–9 vs. 6) and Fuc-Hpt levels were significantly associated with PSA recurrence ($P = 0.011$ and $P = 0.037$, respectively). Multivariable analysis with GS, Fuc-Hpt levels, PSA levels, age, and the pathological finding of capsular invasion and seminal vesicle involvement revealed that only Fuc-Hpt levels was significantly associated with PSA recurrence ($P = 0.024$) (Table II).

TABLE II. Cox Proportional Hazards Analysis to Predict a Patient's Biochemical Recurrence-Free Survival
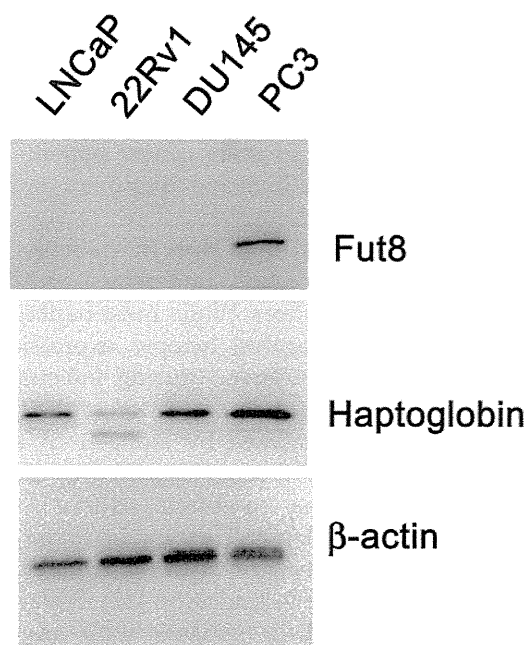
	Univariate		Mutivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age, year	1.06 (0.98–1.14)	0.10		
PSA (ng/dl) (continuous)	1.03 (0.98–1.09)	0.19		
Gleason score (vs. 6)				
7	2.38 (0.66–8.54)	0.18		
8–9	5.84 (1.49–22.9)	0.011		
Capsular invasion	1.22 (0.47–3.17)	0.67		
Seminal vesicle invasion	2.93 (0.85–10.04)	0.087		
Fuc-Hpt (×1,000 U/ml, continuous)	2.65 (1.05–6.66)	0.037	2.905 (1.14–7.34)	0.024

Expression of FUT8 mRNA and Proteins in Prostate Cancer Cell Lines

The origin of elevated serum Fuc-Hpt levels in cancer patients warrant further discussion. It is believed that cancer cells, their surrounding tissues, and infiltrating lymphocytes are all potential candidates for Fuc-Hpt-producing cells. To determine whether haptoglobin and *FUT8* are expressed in prostate cancer cell lines, we first analyzed the mRNA expressions of several kinds of fucosyltransferases by qPCR. As shown in Figure 2, PC3 strongly expressed *FUT8* mRNA, DU145 moderately expressed *FUT8* mRNA, and LNCaP and 22Rv1 weakly expressed *FUT8* mRNA. The mRNA expressions levels of other fucosyltransferases (*FUT1, FUT2, FUT3, FUT4, FUT6, FUT7,* and *FUT9*) were lower in prostate cancer cell lines. Western blots of lysates of LNCaP, 22Rv1, and DU145 revealed weak signals detected by monoclonal

Fig. 3. FUT8 and haptoglobin expression by Western blotting in four prostate cancer cell lines. Total cell lysates from prostate cancer cell lines are immunoblotted using antibodies for FUT8 (upper panel), haptoglobin (middle panel), and beta-actin (lower panel).

antibody against human *FUT8*, and a robust signal in lysates of PC3 (Fig. 3). Robust signals were detected in all four cell lines by monoclonal antibody against human haptoglobin. *FUT8* catalyzes alpha-(1–6)-fucosylation, which contributes to core-type fucosylation. Thus, prostate cancer cells could be a potential source of Fuc-Hpt in patients with prostate cancer.

DISCUSSION

Almost 50% of patients were diagnosed with low-risk prostate cancer in a PSA screening cohort, which would not be life threatening [9]. The overall survival of patients treated with active surveillance for low-risk prostate cancer is comparable to that of patients who undergo prostatectomy. However, approximately 14–16% of the PSA-positive patients undergoing active surveillance experienced disease progression and required definitive therapy [1,2]. Half of the patients with localized prostate cancer had adverse pathology at RP [10,11]. Therefore, the development of biomarkers to detect high-risk prostate cancer and predict prognosis is essential to avoid overtreatment of a disease that is not life threatening or underestimation of aggressive disease. In the present study, we demonstrated that preoperative Fuc-Hpt levels were associated with high GS in RP specimens. PSA and PSAD are reported to be significant predictors of GS upgrade in men diagnosed with low-risk disease [10].

Fig. 2. Expression of fucosyltransferases (*FUT1, FUT2, FUT3, FUT4, FUT6, FUT7, FUT8,* and *FUT9*) in prostate cancer cell lines by quantitative polymerase chain reaction.

The Prostate

We also demonstrated that serum Fuc-Hpt could predict GS upgrade better than PSA and PSAD, although our cohort size was small. The Fuc-Hpt test may prove useful for the prediction of GS upgrade, particularly in low-risk prostate cancer patients who can then undergo less invasive treatment such as active surveillance.

The origin of serum Fuc-Hpt in patients with prostate cancer is still not fully understood, similar to that in other types of cancer. While most haptoglobin is produced by hepatocytes, ectopic expression of haptoglobin has been observed during infection, inflammation, and cancer [4]. Fucosylation of the haptoglobin beta-chain is catalyzed by alpha-(1–3)/alpha-(1–6)-fucosyltransferases. Fucosyltransferases globally modify surface antigens, receptors, adhesion molecules, as well as haptoglobin. They are involved in the regulation of genes associated with malignancy, such as epithelial–mesenchymal transition [12]. Serum Fuc-Hpt level is increased in patients with advanced cancers, including pancreatic cancer, ovarian cancer, lung cancer, and hepatocellular carcinoma [5,13,14]. Serum Fuc-Hpt in colon cancer patients could originate from lymphocytes or hepatocytes around metastatic cancer cells in the liver [7]. Fuc-Hpt production is induced in hepatocytes and hepatoma cells treated with interleukin (IL) 6 [5]. It is hypothesized that hepatocytes around metastatic pancreatic cancer in the liver produce Fuc-Hpt through IL-6 and loss of cellular polarity in metastatic lesions. In prostate cancer, metastasis to the liver is uncommon compared with that to the lymph nodes or bones [15]. However, elevated IL-6 levels were observed in the sera of prostate cancer patients, particularly at advanced stages, with bone metastasis. The majority of the fucosylation was reported as Lewis-type in prostate cancer [8]. We found that prostate cancer cell lines expressed both *FUT8* and haptoglobin, but considerably lower levels of alpha-(1–3)-fucosyltransferase mRNA, and it is suggested that prostate cancer cells mainly secrete core Fuc-Hpt. Lewis-type Fuc-Hpt may be produced by other cells around the prostate cancer.

Expression of *FUT8* is high in several kinds of cancer cells and white blood cells, and very low in normal hepatocytes [4]. A high expression of *FUT8* is observed in non-small cell lung cancer (NSCLC) patients and is associated with poor survival due to upregulation of growth factor-receptor signaling [12]. Increases in *FUT8* expression and core fucosylation of cell surface receptors might promote cell proliferation in high GS prostate cancer. Dysregulation of *FUT8*, with for example, siRNA and/or a neutral antibody for core fucose, might be a possible target for aggressive prostate cancer.

We demonstrated the association of Fuc-Hpt levels with GSs in serum samples obtained before prostatectomy, but not before biopsy. The Fuc-Hpt test may also be useful in a screening setting when combined with the PSA test. Fujimura et al. reported that haptoglobin levels in the sera of prostate cancer patients were significantly higher than those in the sera of patients with benign prostate disease and healthy subjects [8]. The serum samples obtained before biopsy should also be tested to detect intermediate and high-risk prostate cancer. This was a relatively small retrospective cohort study, with a follow-up period of 44 months. Larger scale studies are warranted to confirm these findings.

In conclusion, serum Fuc-Hpt levels were associated with GS, and could be used as a prognostic marker of biochemical recurrence in patients with localized prostate cancer who undergo RP.

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