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ORIGINAL ARTICLE

Percutaneous transhepatic gallbladder drainage followed by elective laparoscopic cholecystectomy in patients with moderate acute cholecystitis under antithrombotic therapy

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Abstract
Background Standard treatment for acute cholecystitis (AC) in patients receiving antithrombotic drugs has not been established. We evaluated the safety of percutaneous transhepatic gallbladder drainage (PTGBD) followed by elective laparoscopic cholecystectomy (LC) in patients with moderate AC who were receiving antithrombotics.
Methods Seventy-five patients received PTGBD from January 2006 to March 2013 followed by elective LC for moderate AC. Patients were divided into Group A, which consisted of patients receiving antithrombotic therapy (n = 23), and Group B, which included the remaining patients (n = 52). We analyzed clinical outcomes and perioperative complications between groups.
Results No hemorrhagic events occurred during PTGBD insertion regardless of antithrombotic treatment. The open conversion rate was not significantly different between the two groups. Postoperative complications were found in 10 patients (13.3%). The rate of postoperative complications in Group A was slightly higher than that in Group B, but the difference was not significant (21.7% vs. 9.6%; P = 0.15). Complications associated with PTGBD occurred in six patients (8%). There were no significant differences in the incidence of these complications, operation time, intraoperative blood loss, or length of postoperative hospital stay.
Conclusions Percutaneous transhepatic gallbladder drainage followed by elective LC may be an effective therapeutic strategy for moderate AC in patients receiving antithrombotic therapy.

Keywords Acute cholecystitis · Antithrombotic therapy · Laparoscopic cholecystectomy · Percutaneous transhepatic gallbladder drainage

Introduction

Acute cholecystitis (AC) is one of the most commonly encountered diseases that is caused by obstruction of the cystic duct with or without gallstones. Early laparoscopic cholecystectomy (LC) has been widely accepted as a standard treatment for patients with AC. Many randomized studies and meta-analyses have indicated the clinical advantage of LC compared to open cholecystectomy (OC) [1, 2] and the value of early LC compared to delayed LC [3, 4]. The Japanese and Tokyo guidelines for acute cholangitis and cholecystitis were published sequentially in 2005 and 2007 [5, 6]. Since that time, early LC has been increasingly used for treating patients with AC [7].

However, there are situations in which surgeons are reluctant to perform early or emergency LC. For example, in elderly or critically ill patients, perioperative mortality rates are high (up to 19%) for emergency cholecystectomy in comparison to cholecystectomy in the elective setting [8]. Even in patients who are not elderly or critically ill, it is sometimes difficult to complete LC because of severe pericholecystic inflammation due to moderate AC

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resulting from various conditions, including biliary peritonitis, pericholecystic abscess, hepatic abscess, gangrenous cholecystitis, and emphysematous cholecystitis. In addition, patients with coagulopathy have increased risks of perioperative thrombotic or hemorrhagic morbidities and mortalities in the emergency or early operative setting. Patients receive oral antiplatelet or anticoagulant therapies to prevent primary or secondary thrombotic cardiovascular or cerebrovascular events, which have increased in incidence due to aging of the population. However, the definitive therapeutic strategy for these patients with AC has not been established.

Percutaneous transhepatic gallbladder drainage (PTGBD) is a less invasive imaging-guided alternative designed to decompress the acutely inflamed gallbladder in patients who are unresponsive to medical therapy or are at high risk for cholecystectomy [9]. Some studies suggest that PTGBD allows subsequent elective cholecystectomy with minimal rates of conversion and perioperative morbidity and mortality in complicated AC [10–15]. This study was designed to evaluate the efficacy and safety of PTGBD followed by elective LC in patients with moderate AC receiving concurrent antithrombotic therapy.

Patients and methods

Patients

We retrospectively reviewed individual medical records from the Hokushinkai Megumino Hospital from January 2006 to March 2013. In this period, 206 patients were diagnosed with AC. The diagnosis of AC was based on clinical signs and findings from computed tomography (CT) scans and ultrasonograms. Severity grading for AC was based on the Japanese guidelines 2005 [5] and the Tokyo guidelines 2007 [16]. Early cholecystectomy including LC and OC is recommended in the Japanese and Tokyo guidelines, but is not adopted in our institute because of insufficient manpower and the lack of a system to do early or emergency operation. Thus, elective cholecystectomy after antibiotics therapy was performed on patients with mild (grade I) AC, and elective cholecystectomy after PTGBD was performed on patients with moderate (grade II) AC or who had not responded to medical therapy. The treatment flow chart according to the therapeutic strategy in our institute was shown in Figure 1. Here, we restricted our study to the patients who underwent elective cholecystectomy after PTGBD. Among them, we analyzed outcomes in 75 patients who received LC, and excluded from analysis 10 patients who received OC, one patient who underwent emergency OC due to failure of PTGBD insertion, and one patient who did not undergo cholecystectomy after PTGBD due to advanced age and poor condition. The distribution of patients according to severity criteria is shown in Table 1.

Antiplatelet or anticoagulant therapy

Twenty-three patients received oral antiplatelet and/or anticoagulant treatment for moderate AC. Thirteen patients received antiplatelet agents, including aspirin and thienopyridines. Seven patients received anticoagulant drugs, and three patients received a combination of antiplatelet and anticoagulant agents. Underlying diseases included ischemic heart disease in 12 patients, atrial fibrillation in seven patients, valvular heart disease in one patient, arteriosclerosis obliterans in two patients, and previous cerebral infarctions in five patients. Patients who were treated with antithrombotic therapy were placed in Group A ($n = 23$), and the remaining 52 patients were placed in Group B. All patients were admitted to our hospital, and oral antiplatelet and/or anticoagulant drugs were immediately discontinued following confirmation of the diagnosis of moderate AC. Nine patients at high risk for cardiovascular or cerebrovascular events needed heparin replacement therapy following discontinuation of oral drugs.

Percutaneous transhepatic gallbladder drainage

Percutaneous transhepatic gallbladder drainage was performed immediately or within a few days after confirming the diagnosis of moderate AC regardless of whether patients received antithrombotic drugs or not. Vitamin K was administered intravenously before PTGBD for one patient who received anticoagulant therapy due to an international normalized ratio of prothrombin time above 2.0. PTGBD was performed according to the Tokyo guidelines [17]. Briefly, an external cylinder with a mandolin was inserted into the gallbladder with ultrasonic guidance. The mandolin was removed, and the external cylinder remained. The backflow of bile was confirmed, and a guide wire was inserted into the gallbladder. The external cylinder was removed. After dilating the track, a 7-Fr drainage tube was passed over the guide wire into the gallbladder. The guide wire was withdrawn and cholangiograms were performed to confirm that the drainage tube was in the correct position within the gallbladder.

Timing for operation and operative technique

Laparoscopic cholecystectomy was electively performed at the appropriate time following PTGBD after the condition of the patient or the pericholecystic inflammation improved. LC was performed at least 7 days after PTGBD in group A, which was the time it took for the antithrombotic effects to

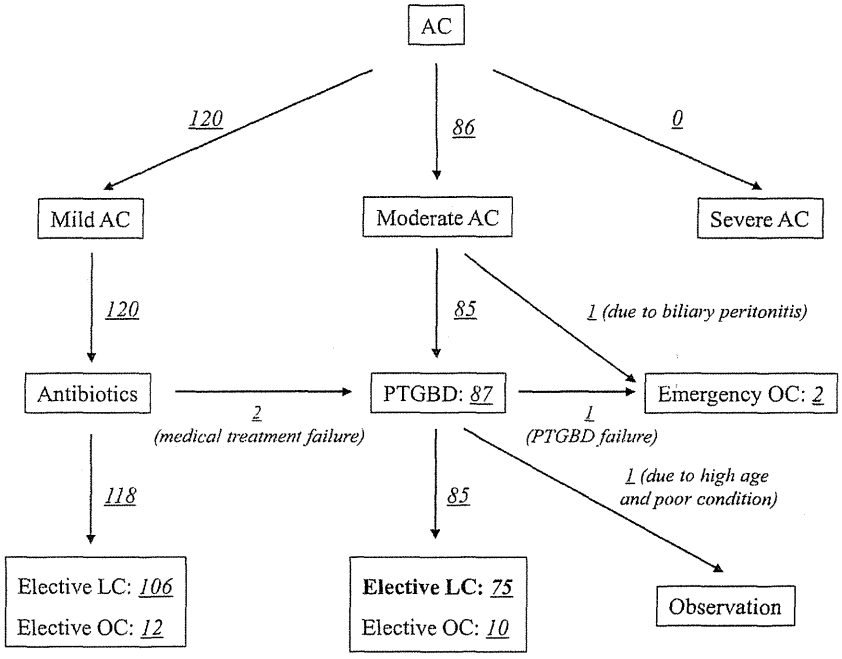

Fig. 1 The flow chart of treatment for acute cholecystitis (AC) according to the strategy in our institute. In our institute, early cholecystectomy including laparoscopic cholecystectomy (LC) and open cholecystectomy (OC) is not adopted. Thus, elective cholecystectomy after antibiotics therapy was performed on patients with mild AC, and elective cholecystectomy after percutaneous transhepatic gallbladder drainage (PTGBD) was performed on patients with moderate AC or who had not responded to medical therapy. Among 120 patients diagnosed with mild AC, two patients were refractory to medical treatment and finally inserted PTGBD. The other patients underwent elective LC in 106 and elective OC in 12. On the other hand, among 86 patients diagnosed with moderate AC, 85 underwent PTGBD, and one underwent emergency OC due to biliary peritonitis. One who failed to PTGBD insertion underwent emergency OC. One who was of advanced age and poor condition did not undergo the operation. The total 85 patients underwent elective cholecystectomy including LC in 75 and OC in 10. In this period, there were no patients diagnosed with severe AC

Table 1 Patient distributions according to severity assessment criteria for acute cholecystitis (AC)

		Group-A (n = 23)	Group-B (n = 52)	P-value
WBC (/μl)	<18,000	18	37	
	≥18,000	5	15	0.584
Palpable tender mass in the right upper abdominal quadrant	No	20	48	
	Yes	3	4	0.669
Duration of complaints	<72 h	18	36	
	≥72 h	5	18	0.579
Pericholecystic abscess	No	18	46	
	Yes	5	6	0.296
Gangrenous cholecystitis	No	20	49	
	Yes	3	5	0.689
Emphysematous cholecystitis	No	20	50	
	Yes	3	2	0.165
Refractory to medical therapy		0	2	0.999
Criteria of "moderate AC" based on the Japanese guideline but not the Tokyo guideline 2007		12	21	0.45

disappear. LC was performed with standard four-trocar technique in the presence of pneumoperitoneum. The PTGBD catheter was removed at the beginning of the operation. After release of inflammatory adhesions around the gallbladder, the triangle of Calot was dissected free of all tissue except for the cystic duct and artery, and the base of the liver bed was exposed. The cystic duct and artery were clipped and transected sequentially. The gallbladder was separated from the liver bed, placed into a disposable plastic bag, and removed from the abdominal cavity. A Penrose drain was inserted for all patients and removed within 24 h if no complications were found.

Statistical analysis

The patient demographics, perioperative characteristics, and rate of perioperative complications were compared between patients who received antiplatelet and/or anticoagulant therapy versus those who did not using the Mann–Whitney test or Fisher's exact test for independence. The data are shown as the median and range. Statistical analysis was performed with StatMate IV for windows (ATMS, Tokyo, Japan), and $P < 0.05$ was considered statistically significant.

Results

The success and response rate of PTGBD

Percutaneous transhepatic gallbladder drainage was performed for 87 patients and was successful in all patients except for one who had severe gangrenous cholecystitis. One patient who failed PTGBD received emergency OC due to poor response to other conservative therapies. All patients in whom PTGBD was successful improved within a few days and were subsequently able to receive elective cholecystectomy except for one who did not undergo operation due to advanced age and poor condition. Accordingly, the success rate and response rate were both 98.9%. Morbidity from PTGBD was 3.5% due to insertion failure in one patient, pleural effusion in another patient, and bile leakage in a third patient. However, the 27 patients who received antithrombotics did not suffer complications from PTGBD, including hemorrhagic events.

Patient demographics and clinical outcomes

In this study, a total of 75 patients were included, and the others were excluded due to OC. The patient demographics and clinical outcomes of PTGBD followed by elective LC are listed in Table 2. The median age was 71 years in Group A and 65 years in Group B ($P < 0.05$). There were no statistical differences in gender, weight, or body mass index (BMI) between Groups A and B. Both groups experienced similar operative times, blood loss, and postoperative clinical outcomes, including length of hospital stay and laboratory test values, such as white blood cell (WBC) count and C-reactive protein (CRP) on postoperative day 3 (Table 2).

Perioperative complications with PTGBD followed by elective LC

Perioperative complications were found in 15 patients (20%; Table 3). No significant differences were found between Groups A and B. Preoperative complications were found in two patients (2.7%), including one patient who developed a pleural effusion in the right thorax and one with bile leakage. Both patients improved with conservative treatment without drainage. Conversion from LC to OC was required in three patients (4%) because of uncontrollable intraoperative bleeding due to severe pericholecystic inflammation and adhesions. However, there were no significant differences between Groups A and B (0% vs. 5.8%; $P = 0.55$). According to the Clavien-Dindo classification [18], postoperative complications occurred in 10 patients (13.3%). Grade II complications occurred in seven patients (10.6%), and grade III occurred in three patients (4%, one with postoperative bleeding and two with bile leakages from the PTGBD route). The patient who developed postoperative bleeding had oozing from the liver bed and required re-operation by laparoscopic surgery. Two patients with bile leakages from the PTGBD route improved with endoscopic nasal biliary drainage and intra-abdominal drainage for several days. The patient who had postoperative subcapsular liver hemorrhage around the route of PTGBD recovered with conservative management after several days. Five patients had persistent postoperative inflammation, defined by high CRP values (more than 10 mg/dl) on postoperative day 3 or continuous fever greater than 37.5°C for more than 3 days. However, we did not find obvious intra-abdominal abscesses by CT scan or ultrasonography, and all five patients recovered with antibiotic therapy. The patient with postoperative pleural effusion improved with conservative therapy. Thus, we did not detect significant differences in outcomes between Group A and B patients, although the incidence of postoperative complications in Group A patients was slightly higher than in Group B patients (21.7% vs. 9.6%; $P = 0.15$). No surgery-related mortalities or serious cardiovascular or cerebrovascular events were observed within 30 days of operation. Complications related to PTGBD were found in six patients (8%, indicated in *Italic font* in Table 3). There were no significant differences in the incidence of these complications between the two patient groups (Table 3).

Table 2 Patient demographics and perioperative characteristics

	Group-A (n = 23)		Group-B (n = 52)		P-value
	Median	Range	Median	Range	
Demographics					
Gender					
Male	16		24		
Female	7		28		0.081
Age (years)	71	(57–95)	65	(22–88)	*0.033*
Height (cm)	164	(140–170)	161	(140–176)	0.438
Weight (kg)	62.7	(52.3–85.0)	62.3	(37.6–93.0)	0.405
BMI (kg/m^2)	24.4	(19.3–35.0)	23.9	(18.6–33.8)	0.346
Preoperative factors					
WBC before PTGBD (/μl)	13310	(6060–24450)	14995	(5770–26470)	0.141
peak CRP before operation (mg/dl)	20.5	(4.1–28.9)	21.795	(0.5–32.7)	0.219
Time interval from onset to PTGBD (days)	2	(0–9)	3	(0–14)	0.158
Time interval from PTGBD to LC (days)	11	(8–23)	12	(4–106)	0.158
Laboratory test after PTGBD					
WBC (/μl)	5680	(3640–10450)	6150	(3500–9890)	0.526
CRP (mg/ml)	1.32	(0.3–10.5)	1.085	(0.1–8.4)	0.280
Intraoperative factors					
Operation time (min)	112	(45–265)	109	(65–180)	0.809
Blood loss (ml)	20	(0–200)	0	(0–840)	0.162
Postoperative factors					
Postoperative hospital stay (days)	4	(4–16)	4	(3–11)	0.425
Laboratory test at 3 postoperative day					
WBC (/μl)	7940	(4420–11750)	6650	(4200–12480)	0.195
CRP (mg/ml)	5.27	(1.3–25.6)	4.505	(0.2–24.5)	0.059

BMI body mass index, *CRP* C-reactive protein, *PTGBD* percutaneous transhepatic gallbladder drainage, *WBC* white blood cell count

Table 3 Perioperative complications after percutaneous transhepatic gallbladder drainage (PTGBD) followed by laparoscopic cholecystectomy (LC)

	Group-A (n = 23)	Group-B (n = 52)	P-value
Total number of complications	5	10	0.532
Preoperative complications	0 (0%)	2 (3.8%)	0.909
Pleural effusion[a]		1	
Intraabdominal bile leakage[a]		1	
Intraoperative complications (conversion to open)	0	3 (5.8%)	0.548
Postoperative complications	5 (21.7%)	5 (9.6%)	0.154
Persistent inflammation after LC	1	4	
Postoperative bleeding (Grade III)	1		
Bile leakage from PTGBD[a] *(Grade III)*	1	1	
Subcapsular hemorrhage of the liver[a]	1		
Pleural effusion[a]	1		
Complication associated with PTGBD	3 (13.0%)	3 (5.8%)	0.363

[a] *Italic* font indicates complications associated with PTGBD

Risk factors associated with complications

Finally, we analyzed the risk factors associated with complications by univariate analysis. Treatment with either antiplatelet or anticoagulant drugs did not increase the incidence of complications. Further, perioperative heparin replacement therapy was not an independent factor. Age older than 65 was an independent risk factor predicting

Table 4 Risk factors associated with perioperative complications

		Complication (n = 15)	Without complication (n = 60)	P-value
Use of anti-platelet or -coagulant agents	Yes	6	17	
	No	9	43	0.532
Use of anti-platelet agents	Yes	4	12	
	No	11	48	0.725
Use of anti-coagulant agents	Yes	1	8	
	No	14	52	0.677
Heparin replacement therapy	Yes	2	7	
	No	13	53	0.859
Gender (male/female)	Male	6	34	
	Female	9	26	0.265
Age (years)	<65	3	30	
	≥65	12	30	*0.045*
BMI (kg/m^2)	<25	9	38	
	≥25	6	22	0.812
Operation time (min)	<120	9	40	
	≥120	6	20	0.763
Blood loss (ml)	<50	9	48	
	≥50	6	12	0.173
Time interval from onset to PTGBD (days)	≤3	10	44	
	>3	5	16	0.749
Time interval from PTGBD to LC (days)	<14	9	42	
	≥14	6	18	0.540
WBC before PTGBD (/μl)	<18,000	13	42	
	≥18,000	2	18	0.328
Peak CRP before LC (mg/ml)	<20	4	26	
	≥20	11	34	0.377

BMI body mass index, *CRP* C-reactive protein, *LC* laparoscopic cholecystectomy, *PTGBD* percutaneous transhepatic gallbladder drainage, *WBC* white blood cell count

perioperative complications from PTGBD followed by elective LC for patients with moderate AC (Table 4).

Discussion

PTGBD is a relatively safe and effective procedure for AC and achieves clinical improvement within 48–72 h after insertion with minimal procedure-related mortality. The review by Itoi et al. showed that the technical success rate and response rate of PTGBD were nearly 100% and 78–95%, respectively. Adverse events related to PTGBD occurred in 0.3–12% of patients [19]. In our series, the success and response rate were nearly 100%. The incidence of complications during PTGBD was 3.5%, and no hemorrhagic events occurred. In addition, Dewhurst et al. have reported that performing percutaneous cholecystostomy in patients with coagulopathy or in those receiving anticoagulant medications did not alter the incidence of hemorrhagic complications in comparison with those who have normal coagulation (1.5% vs. 1.8%) [20]. These data suggest that PTGBD can be performed safely irrespective of the use of antithrombotic drugs.

In patients who received antithrombotic therapy, the conversion rate was 0%, and the rate of postoperative complications was 21.7%. These rates were not different from those observed in patients who did not receive antithrombotic therapy (Table 3). In contrast, hemorrhagic postoperative complications occurred in only two patients receiving antithrombotic therapy. The patient who had postoperative oozing from the liver bed did not suffer any complications during PTGBD insertion, and the duration time from the cessation of aspirin to LC was greater than 14 days. Therefore, the complication was attributed to inflammation from moderate AC and not to PTGBD or aspirin. Another case of subcapsular hemorrhage around the PTGBD route did not occur immediately after insertion of the drainage tube but occurred after the operation. The interval time from the discontinuation of antiplatelets to LC was 10 days. Thus, excessive or forceful intraoperative traction most likely

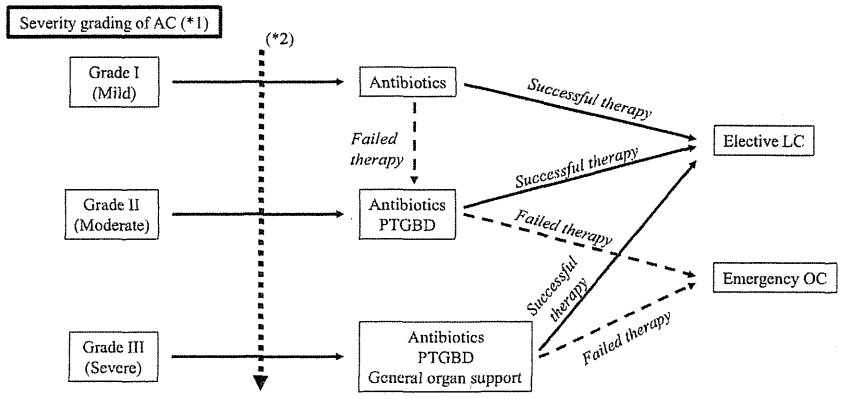

Fig. 2 The treatment strategy for acute cholecystitis (AC) in patients under antithrombotic therapy in our institute. (*1): Severity grading of AC is based on Tokyo guideline 2013. (*2): Antithrombotic drugs are immediately discontinued and heparin replacement is considered if needed. *PTGBD* percutaneous transhepatic gallbladder drainage

induced a slight tear or laceration of the liver parenchyma around the PTGBD route. This is probably related to the PTGBD procedure but not to the influence of antithrombotic therapy. These results suggest that antithrombotic drugs do not increase the risk of perioperative morbidities in PTGBD followed by elective LC for moderate AC.

It is controversial whether PTGBD followed by elective LC can be a standard therapy for moderate AC in non-clinically ill patients. In the 2013 Tokyo guidelines, the indication of early gallbladder drainage and subsequent delayed cholecystectomy, including laparoscopic or open, is restricted to patients with moderate AC who have severe local inflammation [21]. However, the clinical benefits of PTGBD followed by elective LC for complicated AC have recently been shown. The rate of conversion into OC was 3–8%. The incidence rate of postoperative complications was 3.2–16% [10–15], although there have been no randomized controlled studies directly comparing these results with early LC. In our series, the conversion rate and the occurrence rate of postoperative complications was 4% and 13.3%, respectively. These results compared favorably with the above reports. In contrast, perioperative complications associated with PTGBD were found in 8% of patients, including 2.7% with Clavien-Dindo Grade III complications. Our results indicate that it is important to adequately comprehend and pay attention to the particular complications induced by PTGBD if PTGBD followed by LC is performed.

It is assumed that early or emergency LC without PTGBD increases the risk for hemorrhagic events in patients with moderate AC who are receiving antithrombotic therapy due to residual effects from the antithrombotics. This is based in part on the observations that 8.5–27.2% of LC to OC conversions were due to intraoperative bleeding [22–24], and AC significantly increased risk for open conversion and postoperative complications [23–26]. In contrast, by preceding PTGBD, we can wait for the effects of antithrombotics to wear off. In addition, we can appropriately assess the perioperative risk for cardiovascular or cerebrovascular disease during the waiting time. We consider these the greatest benefits of PTGBD followed by elective LC. Thus, this therapeutic strategy seems to be a feasible approach for moderate AC in patients who are receiving antithrombotic therapy. Based on our study, we have developed a new treatment strategy for AC patients with antithrombotic therapy in our institute (Fig. 2). However, our study was small and retrospective, and we did not directly compare our results with those in patients receiving early LC and continued antithrombotic treatment. Further investigation and data accumulation are expected.

Conclusion

The risks of postoperative complications, including severe hemorrhagic complications, were not increased by PTGBD followed by elective LC for moderate AC in patients who received antithrombotic therapy. We therefore conclude that PTGBD followed by elective LC for moderate AC is an acceptable treatment in patients who have received antithrombotic therapy. However, we must pay attention to all PTGBD-related complications, including minor complications.

Conflict of interest None declared.

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Identification of Novel Serum Biomarkers of Hepatocellular Carcinoma Using Glycomic Analysis

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The altered *N*-glycosylation of glycoproteins has been suggested to play an important role in the behavior of malignant cells. Using glycomics technology, we attempted to determine the specific and detailed *N*-glycan profile for hepatocellular carcinoma (HCC) and investigate the prognostic capabilities. From 1999 to 2011, 369 patients underwent primary curative hepatectomy in our facility and were followed up for a median of 60.7 months. As normal controls, 26 living Japanese related liver transplantation donors were selected not infected by hepatitis B and C virus. Their mean age was 40.0 and 15 (57.7%) were male. We used a glycoblotting method to purify *N*-glycans from preoperative blood samples from this cohort (10 μL serum) which were then identified and quantified using mass spectrometry (MS). Correlations between the *N*-glycan levels and the clinicopathologic characteristics and outcomes for these patients were evaluated. Our analysis of the relative areas of all the sugar peaks identified by MS, totaling 67 *N*-glycans, revealed that a proportion had higher relative areas in the HCC cases compared with the normal controls. Fourteen of these molecules had an area under the curve of greater than 0.80. Analysis of the correlation between these 14 *N*-glycans and surgical outcomes by univariate and multivariate analysis identified G2890 (*m/z* value, 2890.052) as a significant recurrence factor and G3560 (*m/z* value, 3560.295) as a significant prognostic factor. G2890 and G3560 were found to be strongly correlated with tumor number, size, and vascular invasion. *Conclusion*: Quantitative glycoblotting based on whole serum *N*-glycan profiling is an effective approach to screening for new biomarkers. The G2890 and G3560 *N*-glycans determined by tumor glycomics appear to be promising biomarkers for malignant behavior in HCCs. (HEPATOLOGY 2013;57:2314-2325)

Hepatocellular carcinoma (HCC) is a common and fatal malignancy with a worldwide occurrence.[1] Liver resection has shown the highest level of control among the local treatments for HCC and is associated with a good survival rate.[2,3] However, the recurrence rates for HCC are still high even when a curative hepatectomy is performed.[4] Many factors associated with the prognosis and recurrence of HCC have now been reported. Vascular invasion of the portal vein and/or hepatic vein and tumor differentiation are important factors affecting survival and recurrence in HCC cases after a hepatectomy.[5,6] However, microvascular invasion and differentiation can only be detected by pathological examination just after a hepatectomy, and cannot be diagnosed preoperatively, and thus cannot be identified preoperatively either. Hence, the serum biomarkers alpha-fetoprotein (AFP) and protein induced by vitamin K absence-II (PIVKA-II) are used as prognostic markers[7,8] and also as surrogate markers for microvascular invasion and tumor differentiation.[9,10] AFP is associated with grade differentiation,[11] whereas PIVKA-II is related to vascular

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; AUC, area under the curve; DFS, disease-free survival; HCC, hepatocellular carcinoma; ICGR15, indocyanin green retention rate at 15 minutes; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; PS, patient survival; RF, risk factor; ROC, receiver operating characteristics.

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invasion.[12,13] However, these tumor markers have limited sensitivity and are less predictive than microvascular invasion,[14,15] which is the most potent determinant of recurrence and survival in HCC patients undergoing a hepatectomy.[5] Therefore, new biomarkers that are more strongly associated with prognosis and recurrence in HCC than AFP or PIVKA-II are highly desirable.

Glycosylation is one of the most common posttranslational protein modifications. Alterations in the *N*-glycosylation profiles of glycoproteins have been suggested to play important roles in the proliferation, differentiation, invasion, and metastasis of malignant cells. Glycan species can be analyzed and characterized using mass spectrometry (MS) and the profiling of these molecules when they are secreted or shed from cancer cells is also performed. Hence, some glycoproteins have been suggested as biomarkers of human carcinomas such as ovarian cancer, breast cancer, and HCC.[16-19] Of note, changes to the N-linked glycan modification of glycoproteins occur during the tumorigenesis and progression of HCC lesions. However, the correlation between the *N*-glycan profile and tumor-associated characteristics such as the degree of malignancy and prognosis has not been previously evaluated in HCC. Recently, we developed a novel glycomics method that facilitates high-throughput and large-scale glycome analysis using an automated glycan purification system, SweetBlot. This approach enables us to profile serum *N*-glycans quantitatively. Using this quantitative *N*-glycomics procedure by way of glycoblotting technology, which is both highly accurate and can be conducted on a large scale, we have previously evaluated the potential of using *N*-glycans as markers of the prognosis and recurrence of HCC.[20]

In our current study we evaluated preoperative blood samples from an HCC patient cohort from which we purified serum *N*-glycans using our glycoblotting method.[21,22] We performed *N*-glycan profiling using MS to search for factors related to prognosis and recurrence by analysis of patient outcomes in 369 consecutive HCC cases that had undergone a primary curative hepatectomy at our medical facility. Through this screen we sought to correlate *N*-glycan levels on glycoproteins with the clinicopathologic characteristics and the outcomes of HCC.

Patients and Methods

Patients. Between April 1999 and March 2011, 369 consecutive adult patients underwent a hepatectomy procedure for HCC at our center and this sample population was examined in the current study. Patients with extrahepatic metastases had been excluded from this cohort because the outcomes of a hepatectomy in these cases are typically very poor. The mean age of the patients in the final study group was 62.7 ± 10.6 years (range, 33-90), 301/369 (81.6%) cases were male, 176 (47.7%) were hepatitis B virus surface antigen-positive, 119 (32.2%) were hepatitis C virus antibody-positive, and 120 (32.5%) were designated as F4 based on the New Inuyama Classification system.[23] The preoperative serum AFP and PIVKA-II levels were simultaneously measured in the patients using standard methods at least 2 weeks before the hepatectomy at the time of the imaging studies. Among the 369 patients in the cohort, 358 (97.0%) were categorized as Child-Pugh class A. According to the TNM stage revised by the Liver Study Group of Japan in 2010,[24] 26 (7.0%) patients were in stage I, 172 (46.6%) in stage II, 111 (30.1%) in stage III, and 60 (16.3%) in stage IVA. The patients were followed up for a median of 60.7 months (range, 9.8-155.1). As a normal control group, 26 living related liver transplantation donors were selected. They were evaluated for eligibility as donors by liver function tests, measurements of the tumor markers AFP and PIVKA-II, and also by x-ray photographs of chest and abdomen and dynamic computed tomography (CT). Their mean age was 40.0 with a range of 20-48. Of 26 controls, 15 (57.7%) were male and 11 (42.3%) were female. All controls were Japanese and not infected by hepatitis B and C virus. This study was approved by the Institutional Review Board of the Hokkaido University, School of Advanced Medicine. Informed consent was obtained from each patient in accordance with the Ethics Committees Guidelines for our institution.

Experimental Procedures: Serum N-Glycomics by Way of Glycoblotting. *N*-glycans from serum samples were purified by glycoblotting using BlotGlycoH. These are commercially available synthetic polymer beads with high-density hydrazide groups (Sumitomo Bakelite,

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Tokyo, Japan). All procedures used the SweetBlot automated glycan purification system containing a 96-well plate platform (System Instruments, Hachioji, Japan).

Enzymatic Degradation of Serum N-Glycans. Each 10-μL serum sample aliquot was dissolved in 50 μL of a 106-mM solution of ammonium bicarbonate containing 12 mM 1,4-dithiothreitol and 0.06% 1-propanesulfonic acid, 2-hydroxyl-3-myristamido (Wako Pure Chemical Industries, Osaka, Japan). After incubation at 60°C for 30 minutes, 123 mM iodoacetamide (10 μL) was added to the mixtures followed by incubation in the dark at room temperature to enable reductive alkylation. After 60 minutes, the mixture was treated with 200 U of trypsin (Sigma-Aldrich, St. Louis, MO) at 37°C for 2 hours, followed by heat-inactivation of the enzyme at 90°C for 10 minutes. After cooling to room temperature, the *N*-glycans were released from the tryptic glycopeptides by incubation with 325 U of PNGase F (New England BioLabs, Ipswich, MA) at 37°C for 6 hours.

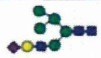
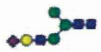
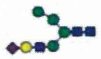
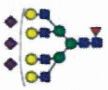
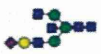
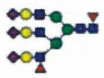
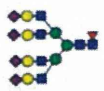
N-Glycan Purification and Modification by Glycoblotting. Glycoblotting of sample mixtures containing whole serum *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (500 μL) (10 mg/ml suspension; Sumitomo Bakelite) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic PTFE (polytetrafluoroethlene) 96-well filter plate (EMD Millipore, Billerica, MA). After removal of the water using a vacuum pump, 20 μL of PNGase F-digested samples were applied to the wells, followed by the addition of 180 μL of 2% acetic acid in acetonitrile. The filter plate was then incubated at 80°C for 45 minutes to capture the *N*-glycans onto the beads by way of a chemically stable and reversible hydrazone bond. The beads were then washed using 200 μL of 2 M guanidine-HCl in 10 mM ammonium bicarbonate, followed by washing with the same volume of water and of 1% triethyl amine in methanol. Each washing step was performed twice. The *N*-glycan linked beads were next incubated with 10% acetic anhydride in 1% triethyl amine in methanol for 30 minutes at room temperature so that unreacted hydrazide groups would become capped by acetylation. After capping, the reaction solution was removed under a vacuum and the beads were serially washed with 2 × 200 μL of 10 mM HCl, 1% triethyl amine in methanol, and dioxane. This is a pretreatment for sialic acid modification. On-bead methyl esterification of carboxyl groups in the sialic acids was carried out with 100 μL of 100 mM 3-methyl-1-*P*-tolyltriazene (Tokyo Chemical Industry, Tokyo, Japan) in dioxane at 60°C for 90 minutes to dryness. After methyl esterification of the more stable glycans, the beads were serially washed in 200 μL of dioxane, water, 1% triethyl amine in methanol, and water. The captured glycans were then subjected to a *trans*-iminization reaction with BOA (O-benzylhydroxylamine) (Tokyo Chemical Industry) reagent for 45 minutes at 80°C. After this reaction, 150 μL of water was added to each well, followed by the recovery of derivatized glycans under a vacuum.

Matrix-Assisted Laser Desorption Ionization, Time-of-Flight (MALDI-TOF) and TOF/TOF Analysis. The *N*-glycans purified by glycoblotting were directly diluted with α-cyano-4-hydroxycinnamic acid diethylamine salt (Sigma-Aldrich) as ionic liquid matrices and spotted onto the MALDI target plate. The analytes were then subjected to MALDI-TOF MS analysis using an Ultraflex time-of-flight mass spectrometer III (Brucker Daltonics, Billerica, MA) in reflector, positive ion mode and typically summing 1,000 shots. The *N*-glycan peaks in the MALDI-TOF MS spectra were selected using FlexAnalysis v. 3 (Brucker Daltonics). The intensity of the isotopic peak of each glycan was normalized using 40 μM of internal standard (disialyloctasaccharide, Tokyo Chemical Industry) for each status, and its concentration was calculated from a calibration curve using human serum standards. The glycan structures were estimated using the GlycoMod Tool (http://br.expasy.org/tools/glycomod/), so that our system could quantitatively measure 67 *N*-glycans.

Hepatectomy. Anatomical resection is defined as a resection in which lesion(s) are completely removed on the basis of Couinaud's classification (segmentectomy, sectionectomy, and hemihepatectomy or more) in patients with a tolerable functional reserve. Nonanatomical partial, but complete resection was achieved in all of our cases. R0 resections were performed while the resection surface was found to be histologically free of HCC. The indocyanin green retention rate at 15 minutes was measured in each case to evaluate the liver function reserve, regardless of the presence or absence of cirrhosis.

HCC Recurrence. For the first 2 years after the hepatectomy procedure, the HCC patients in our cohort were monitored every 3 months using liver function tests, measurements of the tumor markers AFP and protein induced by PIVKA-II, and also by ultrasonography and dynamic CT. At 2 years postsurgery, routine CT was performed only once in 4 months. If recurrence was suspected, both CT and magnetic resonance imaging (MRI) were performed and, if necessary, CT during angiography and bone scintigraphy were undertaken.

Table 1. List of the 14 Serum N-Glycans That Were Evaluated to be Specific for Hepatocellular Carcinoma Compared with Normal Controls by Receiver Operating Characteristic (ROC) Analysis

N-glycans	m/z		Specificity (%)	Sensitivity (%)	Cutoff Value	AUC
G2032	2032.724		100	86.45	1.115	0.968
G2890	2890.052		92.31	82.66	0.844	0.91
G1793	1793.672		92.31	75.61	1.963	0.9
G1708	1708.619		88.46	77.51	0.604	0.896
G1870	1870.672		88.46	75.88	2.886	0.873
G1955	1955.724		100	59.89	3.913	0.873
G3195	3195.163		92.31	71.27	6.109	0.864
G3560	3560.295		88.46	71.27	0.091	0.851
G2114	2114.778		88.46	75.88	2.208	0.839
G1809	1809.666		84.62	72.9	0.679	0.838
G3341	3341.221		84.62	69.92	0.086	0.821
G1590	1590.592		80.77	69.92	10.696	0.817
G1362	1362.481		65.38	87.26	1.381	0.813
G3865	3865.407		92.31	56.37	0.121	0.812

The area-under-the-curve (AUC) values of these 14 serum N-glycan were greater than 0.80. These glycan structures are represented with the symbol nomenclature explained in http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml.

This enabled a precise diagnosis of the site, number, size, and invasiveness of any recurrent lesions.

Statistics. The specificity, the sensitivity, cutoff, and AUC (area under the curve) values of selected *N*-glycans are shown in Table 1. This ROC (receiver operating characteristics) analysis was carried out using R v. 2.12.1. The patient survival (PS) and disease-free survival rates (DFS) were determined using the Kaplan-Meier method and compared between groups by the log-rank test. Univariate analysis of variables was also performed, and selected variables using Akaike's Information Criterion (AIC)[25] were analyzed with the Cox proportional hazard model for multivariate analysis. Statistical analyses were performed using

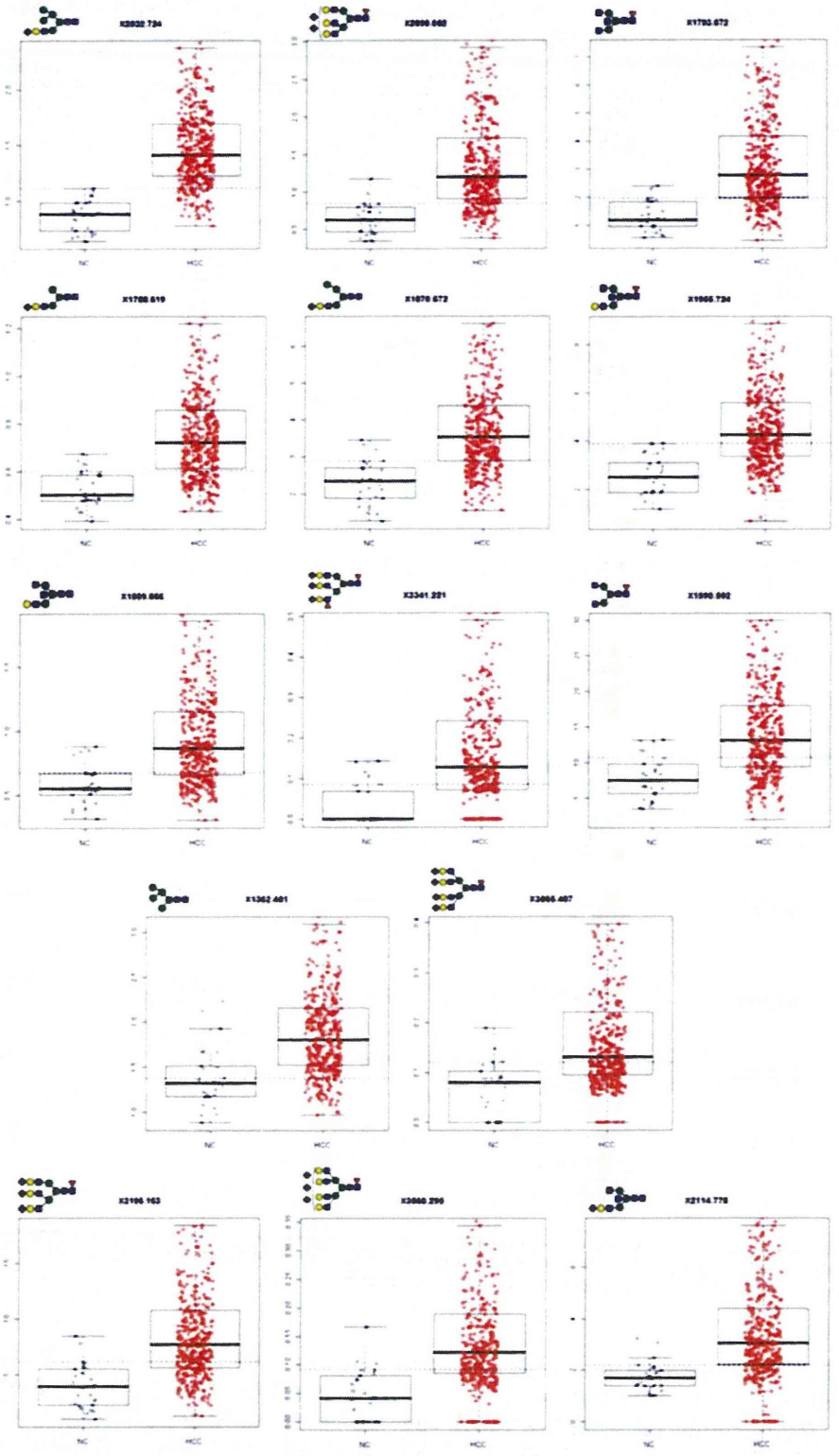


Fig. 1. Boxplots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cutoff values determined in this analysis. These graphs were drawn using R v. 2.12.1.

Table 2. Univariate Analysis of Predictive Values (the Selected 14 *N*-Glycans) of Patient Survival (PS) and Disease-Free Survival (DFS)

		(n)	PS Hazard Ratio	PS *P*-value	DFS Hazard Ratio	DFS *P*-value
G2032	Low	206	1	0.9362	1	0.1054
	High	163	1.017		1.243	
G2890	Low	152	1	<0.0001	1	0.0001
	High	217	3.044		1.705	
G1793	Low	112	1	0.6829	1	0.2897
	High	257	1.095		1.168	
G1708	Low	145	1	0.0016	1	0.0043
	High	224	2.017		1.485	
G1870	Low	151	1	0.5552	1	0.4008
	High	218	1.132		1.122	
G1955	Low	113	1	0.4213	1	0.795
	High	256	1.2		1.038	
G3195	Low	206	1	<0.0001	1	0.0001
	High	163	3.238		1.662	
G3560	Low	246	1	<0.0001	1	<0.0001
	High	123	4.209		1.74	
G2114	Low	275	1	0.0056	1	0.1627
	High	94	1.776		1.232	
G1809	Low	238	1	0.0027	1	0.055
	High	131	1.824		1.306	
G3341	Low	188	1	<0.0001	1	0.0005
	High	181	3.185		1.592	
G1590	Low	167	1	0.0956	1	0.9102
	High	202	1.413		0.985	
G1362	Low	261	1	0.0399	1	0.0004
	High	108	1.526		1.634	
G3865	Low	192	1	<0.0001	1	0.0014
	High	177	3.145		1.532	

standard tests (χ^2, *t* test) where appropriate using StatView 5.0 for Windows (SAS Institute, Cary, NC). Significance was defined as $P < 0.05$.

Results

Profiling of Human Serum Glycoforms and ROC Analysis in HCC Patients and Normal Controls. *N*-glycan profiles of blood samples from our HCC cohort were obtained by MALDI-TOF MS analysis using the high-throughput features of the instrument. We thereby identified 67 *N*-glycans from which we selected molecules that showed statistical differences by ROC analysis between HCC and disease-free individuals (normal controls, NC) comprising living related liver transplantation donors. Glycans with an AUC value greater than 0.80 were selected for analysis (Table 1) and boxplots for these selected molecules (14 in total) are shown in Fig. 1. Clear differences in the distribution of these factors are evident between the NC and HCC patients. The cutoff values were determined using the maximum values for specificity plus sensitivity. G2890 was elevated more than a cutoff value in 305 (82.7%) of HCC patients and G3560 in 261 (70.7%).

Causes of Death. There were 115 deaths in total among our 369 HCC patient cohort (31.2%). The causes of death were as follows: HCC recurrence (n = 97; 84.3%), liver failure (n = 6; 5.2%), and other causes (n = 12; 10.4%).

Univariate Analysis and Multivariate Analysis of Overall Patient and Disease-Free Survival. The overall PS rates at 1, 3, and 5 years in our HCC cohort were 88.8%, 76.4%, and 67.6%, respectively. The DFS values for this groups at 1, 3, and 5 years were 64.0%, 35.5%, and 27.4%, respectively. The 14 serum N-glycans that were highly specific for HCC were evaluated for 3-year recurrence-free survival by ROC analysis to determine the cutoff values about these N-glycans. The patients were divided to two groups by these cutoff values. The PS and DFS measurements associated with the selected 14 selected *N*-glycans were evaluated by univariate analysis. The *P* values for the PS rates associated with G2890, G1708, G3195, G3560, G2114, G1809, G3341, G1362, and G3865 were all less than 0.05. The DFS *P* values for G2890, G1708, G3195, G3560, G3341, G1362, and G3865 were also less than 0.05 (Table 2). When clinical and tumor-associated factors were evaluated by univariate analysis, albumin, Child-Pugh classification,

Table 3. Univariate Analysis of Predictive Values (Clinical and Tumor Associated Factors) for Patient Survival (PS) and Disease-Free Survival (DFS)

		(n)	PS Hazard Ratio	PS *P*-value	DFS Hazard Ratio	DFS *P*-value
Sex	Male	301	1	0.7486	1	0.6535
	Female	68	0.913		0.943	
Age (years)	<=62	160	1	0.3272	1	0.6320
	62<	209	1.211		1.106	
HBV	Positive	176	1.259	0.1911	1.007	0.8093
	Negative	192	1		1	
HCV	Positive	119	1.291	0.2433	1.008	0.8183
	Negative	250	1		1	
Albumin (mg/dL)	<=4.05	147	2.128	<0.0001	1.626	0.0001
	4.05<	222	1		1	
Total bilirubin (mg/dL)	<=0.82	235	1	0.5831	1	0.5241
	0.82<	134	1.122		1.128	
ICGR15 (%)	<=16.7	223	1	0.1223	1	0.0106
	16.7<	146	1.349		1.375	
Child-Pugh	A	358	1	<0.0001	1	0.0374
	B	11	4.292		2.169	
Anatomical resection	Anatomical	282	1	0.8569	1	0.1435
	Nonanatomical	87	0.949		1.225	
AFP (ng/mL)	<=20	183	1	<0.0001	1	0.0008
	20<<=1000	115	2.395		1.449	
	1000<	71	4.433		1.870	
AFP-L3 (%)	<=15	255	1	<0.0001	1	0.0567
	15<	113	2.366		1.285	
PIVKA-II (mAU/mL)	<=40	109	1	<0.0001	1	0.0095
	40<<=1000	133	1.593		1.240	
	1000<	123	3.784		1.635	
Number	Single	235	1	<0.0001	1	<0.0001
	2,3	89	3.731		2.252	
	4<=	45	7.299		3.788	
Size (cm)	<=3	116	1	<0.0001	1	0.0086
	3<<=5	96	2.688		1.260	
	5<	157	4.049		1.570	
Differentiation	Well	17	1	0.0003	1	0.0002
	Moderately	190	2.568		2.990	
	Poorly	159	5.358		4.361	
Vp	Positive	94	4.630	<0.0001	2.156	<0.0001
	Negative	275	1		1	
Vv	Positive	35	5	<0.0001	1.969	0.0004
	Negative	334	1		1	
Macroscopic vascular invasion	Positive	48	6.135	<0.0001	1.961	<0.0001
	Negative	321	1		1	
Stage	1	26	1	<0.0001	1	<0.0001
	2	172	2.844		1.206	
	3	111	9.901		2.404	
	4A	60	15.625		3.106	
Noncancerous liver	Cirrhosis	120	1.199	0.3105	1.293	0.0398
	Noncirrhosis	249	1		1	

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein; vv, microscopic tumor thrombus in the hepatic vein; HBV, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICGR15, indocyanin green retention rate at 15 minutes.

AFP, AFP-L3 (lens culinaris agglutinin-reactive fraction of alpha-fetoprotein), PIVKA-II, tumor number, tumor size, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, and stage were found to be significantly associated with the PS rate. When the same analysis was undertaken for the DFS rate by univariate analysis, albumin, indocyanin green retention rate at 15 minutes, Child-Pugh classification, AFP, PIVKA-II, tumor number, tumor size, differentiation, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, stage, and noncancerous liver were found to be significantly associated with this measure (Table 3).

The variable selection from 19 clinical and tumor-associated factors in Table 3 and the 14 serum

Table 4. Multivariate Analysis of Values That Is Predictive for Overall HCC Patient Survival

		P	Hazard Ratio	95% Confidence Interval	
ICGR15 (%)	16.7<	0.000209	2.435	1.5213	3.898
Child-Pugh	B	0.011136	3.007	1.2852	7.037
AFP (ng/mL)	20<<=1000	0.0003	2.558	1.5372	4.256
	1000<	0.000217	2.782	1.6177	4.786
Tumor number	2,3	0.011844	1.937	1.1575	3.241
	4<=	<0.0001	2.989	1.7693	5.049
Size (cm)	3<<=5	0.278625	1.483	0.7269	3.026
	5<	0.016071	2.237	1.1613	4.307
Vp	Positive	<0.0001	2.982	1.8446	4.822
C3560	>0.158	<0.0001	2.52	1.6191	3.923

ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

Table 5. Multivariate Analysis of Values That Are Predictive of Disease-Free Survival in HCC Patients

		P	Hazard Ratio	95% Confidence Interval	
ICGR15 (%)	16.7<	0.00334	1.519	1.149	2.008
AFP (ng/mL)	20<<=1000	0.04904	1.366	1.001	1.864
	1000<	0.01851	1.591	1.081	2.342
Tumor number	2,3	0.0072	1.551	1.126	2.135
	4<=	<0.0001	2.649	1.704	4.118
Differentiation	Moderately	0:01495	2.838	1.225	6.577
	Poor	0.00501	3.398	1.446	7.984
vp	Positive	0.01023	1.544	1.108	2.152
C2890	>1.12	0.01125	1.443	1.087	1.915

ICGR15, indocyanin green retention rate at 15 minutes, AFP, alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein.

N-glycans using the AIC was performed and the selected valuables were analyzed with PS and DFS by multivariate analysis. G3560 were found to be independent risk factors for PS (Table 4) and G2890 for DFS (Table 5).

The PS rates of HCC cases with low serum G3560 levels at 5 years were 80.5% and of high serum G3560 at 5 years were 40.4%. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively (Fig. 2).

Relationship Between Clinical and Tumor-Associated Factors in HCC and Specific Glycans. Among the low and high G2890 HCC groups, there were significant differences found in a number of clinical and tumor-associated factors including albumin, Child-Pugh classification, AFP, PIVKA-II, tumor number, tumor size, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, and stage (Table 6). In comparing the low and high G3560 HCC patients, significant differences were found in albumin, Child-Pugh Classification, operative procedures, AFP, AFP-L3, PIVKA-II, tumor number, tumor size, differentiation profiles, microscopic portal vein invasion, microscopic hepatic vein invasion, macroscopic vascular invasion, and stage (Table 6).

Discussion

The *N*-glycan profiles of a large cohort of HCC patients were obtained in our current study by MALDI-TOF MS analysis and 67 of these molecules were thereby quantified. Of this group of factors, 14 *N*-glycans showed higher relative peaks in the HCC patients compared with normal controls and were

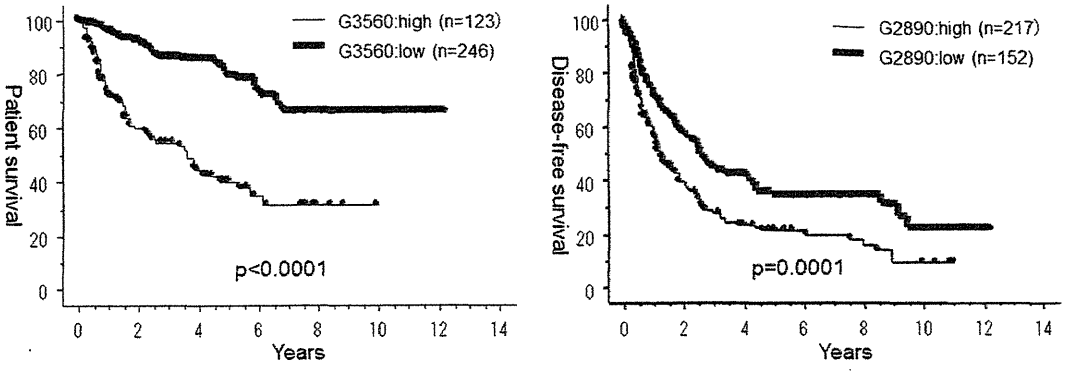


Fig. 2. The PS rates of HCC cases with low and high serum G3560 levels at 5 years were 80.5% and 40.4%, respectively. The DFS outcomes associated with low and high serum G2890 levels at 5 years were 21.3% and 35.1%, respectively.

Table 6. Correlation Between the G2890 and G3560 *N*-Glycans and Clinical and Tumor Associated Factors in HCC Cases

		G2890			G3560		
		High (n=217)	Low (n=152)	P	High (n=123)	Low (n=246)	P
Sex	Male	184	117	0.0767	105	196	0.2286
	Female	33	35		18	50	
Age	≦62	90	70	0.4433	49	111	0.393
	>62	127	82		74	135	
HBV	Positive	107	69	0.5254	59	117	0.9706
	Negative	110	83		64	129	
HCV	Positive	63	56	0.1425	32	87	0.0904
	Negative	154	96		91	159	
Albumin (mg/dL)	≦4.05	109	38	<0.0001	73	74	<0.0001
	>4.05	108	114		50	172	
Total bilirubin (mg/dL)	≦0.82	136	99	0.7088	82	153	0.4671
	>0.82	81	53		41	93	
ICGR15 (%)	≦16.7	125	98	0.2224	77	146	0.6246
	>16.7	92	54		46	100	
Child-Pugh	A	206	152	0.0034	115	243	0.008
	B	11	0		8	3	
Anatomical resection	Anatomical	172	110	0.1583	106	176	0.0028
	Nonanatomical	45	42		17	70	
AFP (ng/mL)	≦20	102	81	0.0461	52	131	<0.0001
	20< & ≦1000	64	51		30	85	
	>1000	51	20		41	30	
AFP-L3 (%)	≦15	143	112	0.1147	68	187	<0.0001
	>15	74	40		55	59	
PIVKA II (mAU/mL)	≦40	52	58	0.0001	22	88	<0.0001
	40< & ≦1000	74	60		33	101	
	>1000	91	34		68	57	
Number	Single	122	113	0.0009	68	167	<0.0001
	2, 3	60	29		27	62	
	≧4	35	10		28	17	
Size (cm)	≦3	48	68	<0.0001	15	101	<0.0001
	3< & ≦5	60	36		21	75	
	>5	109	48		87	70	
Differentiation	Well	12	8	0.0981	6	14	0.0003
	Moderately	102	88		46	144	
	Poorly	103	56		71	88	
vp	Positive	67	27	0.0065	49	45	<0.0001
	Negative	150	125		74	201	
vv	Positive	29	6	0.0043	24	11	<0.0001
	Negative	188	146		99	235	
Macroscopic vascular invasion	Positive	43	5	<0.0001	32	16	<0.0001
	Negative	174	147		91	230	
Stage	1	7	19	<0.0001	3	23	<0.0001
	2	88	84	45	127		
	3	71	40	35	76		
	4A	51	9	40	20		
Noncancerous liver	Cirrhosis	71	49	0.9876	35	85	0.2888
	Noncirrhosis	146	103		88	161	

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonism factor II; AFP-L3, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; vp, microscopic tumor thrombus in the portal vein; vv, microscopic tumor thrombus in the hepatic vein; HBV, hepatitis B virus s antigen; HCV, anti-hepatitis C virus antibody; ICGR15, indocyanin green retention rate at 15 minutes.

chosen for further analysis. These selected molecules were assessed for any correlation with surgical outcomes in the HCC cohort (i.e., prognosis and recurrence) by univariate and multivariate analysis. G3560 *N*-glycan was found to be a significant prognostic factor and G2890 *N*-glycan was found to be a significant recurrence factor for this disease. Moreover, G2890 and G3560 were found to strongly correlate with a number of well-known tumor-related prognostic and recurrent factors. These results show that quantitative glycoblotting based on whole serum *N*-glycan profiling is a potent screening approach for novel HCC biomarkers, and that the G3560 and G2890 *N*-glycans are promising biomarkers of the PS, DFS, and malignant behavior characteristics of HCC after hepatectomy.

Although glycans, once released from glycoproteins or glycopeptides, have been subjected to fluorescent labeling and purification for detection by high-performance liquid chromatography (HPLC) previously, this method is time-consuming and therefore not suited to clinical diagnosis. Our novel analytical method, which we refer to as glycoblotting, is far more rapid and accurate, as evidenced by the number of *N*-glycans detected in our current analysis. This chemoselective glycan enrichment technology known as glycoblotting was developed in our laboratory to purify oligosaccharides derived from glycoproteins in an effective and quantitative manner, thus enabling serum glycan profiling by way of a simpler method.[20] Our method is also applicable to the fully automated analysis of multiple samples simultaneously. It readily combines the isolation and labeling of oligosaccharides, which can then be subjected to conventional analytical methods including MS. We had already achieved high-speed quantitative and qualitative profiling of glycan expression patterns in biological materials using this technology. In our present study, we improved the method to allow quantitative analysis of high reproducibility and accuracy using a calibration curve of human serum standards. The analysis of the obtained 67 glycan profiles was performed using this new developed technology. The effectiveness of our method is evidenced by the identification of the G2890 and G3560 *N*-glycans as highly promising clinical markers of HCC associated with the PS, DFS, and tumor malignancy rates of these cancers.

It has been reported that AFP is the most significant tumor marker and independent predictor of prognosis for HCC,[26] even in patients who have received a hepatectomy.[27] Although high levels of AFP in cases of fully developed HCC, or in the serum of the host, are known to be associated with more aggressive behavior, and increased anaplasis,[28] AFP can also cause apoptosis in tumor cells.[29] Moreover, it has been suggested that AFP regulates the immune response and induces either stimulatory or inhibitory growth activity.[30] On the other hand, it is well known that AFP may increase in some patients with acute and chronic hepatitis without HCC,[31,32] and that the elevation of AFP correlates with inflammation of background disease and hepatocyte regeneration.[33] Hence, because the AFP profile does not always directly reflect the extent of tumor malignancy, the AFP levels do not influence patient survival and recurrence. On the other hand, AFP and many important tumor markers, such as carcinoembryonic antigen, carbohydrate antigen 125, and carbohydrate antigen 19-9, are glycoproteins, and this means that the glycan profiles in serum are altered by the onset of cancer. Indeed, the profiling of serum glycans has been performed previously as a screen for distinct potential glycan biomarkers of ovarian cancer and breast cancer.[18,19] Hence, we surmised that highly specific glycoprotein markers of HCC should be detected by monitoring the serum glycosylation profile in these patients. In glycan structure, both G2890 and G3560 are multiply branched (G2890 is tri-antennary and G3560 is tetra-antennary) glycans with a core fucose. In addition, both glycans have one nonsialylated branch, i.e., G2890 and G3560, are tri-antennary disialylated glycan, and tetra-antennary tri-sialylated glycan, respectively. The structure of G2890 and G3560 is quite different from the AFC-L3 (core fucosylated bi-antennary glycan) and CA19-9 (sialylated Lewis (a) antigen), which are well-known biomarkers related to HCC except for the core fucosylation.

There have been several previous studies of glycans in HCC. Kudo et al.[34] reported that *N*-glycan alterations are associated with drug resistance in HCC *in vitro*. In other reported clinical studies, only specific glycans have been assessed in relation to HCC. Vanhooren et al.[17] were the first to analyze the function of HCC-specific glycans, and reported that a triantennary glycan (NA-3Fb) correlated with the tumor stage and AFP levels in HCC patients. However, that study analyzed 44 patients with HCC but did not evaluate the relationship between the *N*-glycans and the clinical and pathological factors of this disease, the clinical course after hepatectomy, or prognosis and recurrence. In our current study, in contrast, we analyzed a far larger cohort than any other previous report, and evaluated a comprehensive panel of clinical and pathological parameters in relation to the *N*-glycan profile in HCC. Tang et al.[35] also described some HCC-specific glycans in their previous study that we did not find to be significant in our current analyses. This is likely due to the fact that the patient number in their study was smaller than ours, and the fact that the *N*-glycome profile in serum is gender- and age-dependent.[36] In this study, the mean age and the distribution of gender and infection of hepatitis B and C virus were the difference between NC and HCC patients. However, the selected 14 serum *N*-glycans were quantified by our MALDI-TOF MS analysis and compared with NC by ROC analysis. These were statistically different between HCC and NC with respect to the quantity. Because these 14 serum *N*-glycans of which the AUC values were greater than 0.80 were revealed to be specific for HCC, they had a high discriminating ability to differentiate HCC from NC. Further analyses are

required to determine whether G2890 and G3560 are elevated in patients with hepatitis B, hepatitis C, and/or cirrhosis without HCC.

The most important adverse prognostic factor for liver resection and transplantation in HCC has been found to be microscopic venous invasion.[5] However, microscopic portal invasion is not diagnosed preoperatively, and is revealed only by pathological examination. New biomarkers that are more strongly associated with prognosis and recurrence of HCC than AFP, AFP-L3, or PIVKA-II are therefore highly desirable. Our current data show that the *N*-glycans G2890 and G3560 correlate closely with well-known tumor-related prognostic and recurrent factors such as tumor number, size, microscopic portal vein invasion, microscopic hepatic vein invasion, differentiation, macroscopic vascular invasion, stage, AFP, AFP-L3, and PIVKA-II (Table 6). Moreover, when G2890 and G3560 were simultaneously included in multivariate analysis for PS and DFS with AFP, AFPL3 and PIVKA-II, *P*-values of G2890 and G3560 were lower than AFP, and AFPL3, and PIVKA-II were not selected as valuables by AIC. We demonstrate that these are novel independent prognostic factors for HCC that are related to the survival and recurrence of this disease and that show a lower *P*-value than other established tumor factors. Hence, we predict that G2890 and G3560 will prove to be markers that can preoperatively predict HCC tumor malignancy including microscopic portal vein invasion, and the PS and DFS rates more accurately and with more potency than the more well-known biomarkers.

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