Table 2 Characteristics of tumor thrombi

Patients age/sex	Involved veins	Extent of thrombus	Advance of thrombus	Symptoms associated with the thrombus
68/M	RHV	Massive	RA	Renal insufficiency/lower limb edema
57/M	RAdV	Massive	IVC	Renal insufficiency
70/M	RHV	IVC occlusive	RA	Renal insufficiency/lower limb edema
86/F	RHV	Massive	IVC	(–)
68/M	IRHV	Mural	IVC	(–)
66/M	RHV	Massive	RA	(–)
37/M	IRHV	Massive	IVC	(–)
56/M	RHV/IRHV	Massive	IVC	(–)
51/M	LHV	Massive	RA	(–)
72/M	MHV	Mural	IVC	(–)
59/M	LHV/MHV	Massive	RA	(–)
69/M	MHV	Massive	IVC	(–)
65/M	MHV	Massive	RA	(–)

M male, *RHV* Right hepatic vein, *RAdV* right adrenal vein, *IRHV* inferior right hepatic vein, *LHV* left hepatic vein, *MHV*, middle hepatic vein, *IVC* inferior vena cava, *RA* right atrium. (–), no symptom.

nodes in three, brain in two, IVC in one, and adrenal gland in 1 (Tables 1 and 5).

The 1-, and 3-year overall survival rates for all 13 patients were 50.4% and 21.0%, respectively, and the overall median survival duration was 15.3 months. The cause of postoperative death in all patients was cancer, which remained at surgery or recurred after surgery (Table 5). The overall survival rate for patients with IVC thrombi was 57.1% at 1 year and 42.9% at 3 years, with median survival duration of 15.3 months. The 1-year overall survival rate for patients with RA thrombi was 40.0%, with median survival duration of 11.2 months. There was no significant difference between the IVC thrombi and RA thrombi groups (Figure 2a). The survival rates for patients who underwent curative surgical resection were 80.0% at 1 year and 30.0% at 3 years, with a median survival time of 30.8 months. Meanwhile, the 1-year survival rate for patients who underwent noncurative surgery and had residual tumors was 29.2%, with a median survival time of 10.5 months (Figure 2b). The longest survival time was 51.8 months for patients who underwent complete resection and 29.3 months (to date) for those who underwent incomplete resection, and they are still alive (Table 5).

Table 3 Surgical procedure

	Inferior vena cava thrombus (n = 7)	Right atrium thrombus (n = 6)
Surgical procedure		
Extended right hepatectomy	1	1
Extended right hepatectomy + right adrenectomy	0	1
Right hepatectomy	3	1
Right hepatectomy + right adrenectomy	1	0
Extended left hepatectomy	1	2
Sectionectomy	1	1
Inflow vascular control		
Hepatic vascular exclusion	7	0
Cardiopulmonary bypass	0	4
CPB + portal vein/inferior vena cava to superior vena cava bypass	0	2
Vascular wall reconstruction		
Simple closure	7	4
Patch reconstruction	0	2

Discussion

IVC and RA tumor thrombi arising from HCC are uncommon and are found in approximately 3 to 4% of HCC patients [2,23]. It is recognized that tumor invasion into intrahepatic vessels, such as the portal or hepatic veins, is an important prognostic factor for patients with HCC [24]. In particular, the prognosis of patients presenting with ICV or RA thrombi is extremely dismal [6]. Although surgical treatments as well as nonsurgical treatments such as TACE, radiotherapy, and chemotherapy are reported, optimal therapeutic management of IVC and RA thrombi has not been established because of the paucity of data [5,7,8,10-16]. Some reports demonstrate the potential benefit of surgical resection, but there are few reports that consolidate the efficacy of a surgical approach because IVC and RA thrombi are rare and because these reports are typically case reports or descriptions of a small number of patients [4,9,16,17,20,25,26]. Reports detailing the surgical treatment of RA thrombi

Table 4 Surgical outcomes

	Inferior vena cava thrombus	Right atrium thrombus
Surgical duration (minutes)		
Mean ± SD (range)	349 ± 30 (288 to 377)	608 ± 169 (449 to 911)
Blood loss (ml)		
Median ± SE (range)	950 ± 100 (750 to 1,520)	6540 ± 5404 (1,050 to 35,820)
Blood transfusion		
Yes/no	2/5	5/1
HVE time (minutes)		
Mean ± SD (range)	8.8 ± 3.1 (8 to 13)	-
CPB time (minutes)		
Mean ± SD (range)	-	32.2 ± 18.3 (4 to 54)
Curative resection		
Yes/no	3/4	2/4
ICU stay (days)		
Mean ± SD (range)	-	1.7 ± 0.8 (0–2)
Hospital stay (days)		
Mean ± SD (range)	23.6 ± 12.5 (14 to 48)	21.2 ± 4.6 (16 to 28)
Complications		
Yes/no	2 (ascites, 1; biloma, 1)/5	2 (ARF, 1; Af, 1)/4

HVE hepatic vascular exclusion, *CPB* cardiopulmonary bypass, *ARF* acute renal failure, *Af* atrial fibrillation.

are particularly rare, and to our knowledge this is the first report on the surgical treatment of IVC and RA thrombi, including six cases of RA thrombectomy, from a single institute.

It is generally assumed that liver resection combined with IVC or RA thrombectomy is a challenging and hazardous procedure that involves a high surgical risk. According to past reports, hepatectomy together with IVC or RA thrombectomy was associated with a high morbidity of 40% and a high mortality of 15% [4,15]. However, recent surgical innovations such as the inflow vascular control method together with refinement of the assessment of preoperative hepatic reserve have improved the safety of hepatectomy and thrombectomy procedures [26,27]. This progress has encouraged us to accept the challenge of aggressive surgical treatment for IVC and RA thrombi.

Effective control of intraoperative hemorrhage plays a crucial role in hepatectomy procedures combined with

Table 5 Characteristics and prognosis of patients

Patients age/sex	Tumor thrombus	Residual tumor		Metastatic recurrence		Outcome (cause of death)
		Hepatic	Distant	Hepatic	Distant	
68/M	RA	(–)	(–)	(+)	(+) (lung, Ad)	30.8 months; dead (cancer)
57/M	IVC	(–)	(–)	(+)	(–)	10.1 months; dead (cancer)
70/M	RA	(+)	(–)		(+) (lung)	9.1 months; dead (cancer)
86/F	IVC	(–)	(–)	(+)	(+) (lung, IVC)	15.3 months; dead (cancer)
68/M	IVC	(–)	(–)	(+)	(+) (lung, LN)	51.8 months; alive
66/M	RA	(–)	(+) (lung)	(+)	(+) (LN)	11.2 months; dead (cancer)
37/M	IVC	(–)	(+) (lung)	(–)	(+) (Ad, LN, Brain)	10.5 months; dead (cancer)
56/M	IVC	(–)	(+) (lung)	(–)	(–)	29.3 months; alive
51/M	RA	(–)	(+) (lung)	(+)	(+) (Brain)	8.5 months; dead (cancer)
72/M	IVC	(+)	(+) (lung)		(+) (LN)	1.9 months; dead (cancer)
59/M	RA	(–)	(–)	(+)	(+) (lung)	16.5 months; alive
69/M	IVC	(–)	(+) (LN)	(+)	(+) (lung, IVC)	16.0 months; alive
65/M	RA	(+)	(–)		(+) (lung)	7.6 months; alive

Ad adrenal gland, *IVC* inferior vena cava, *LN* lymph node.

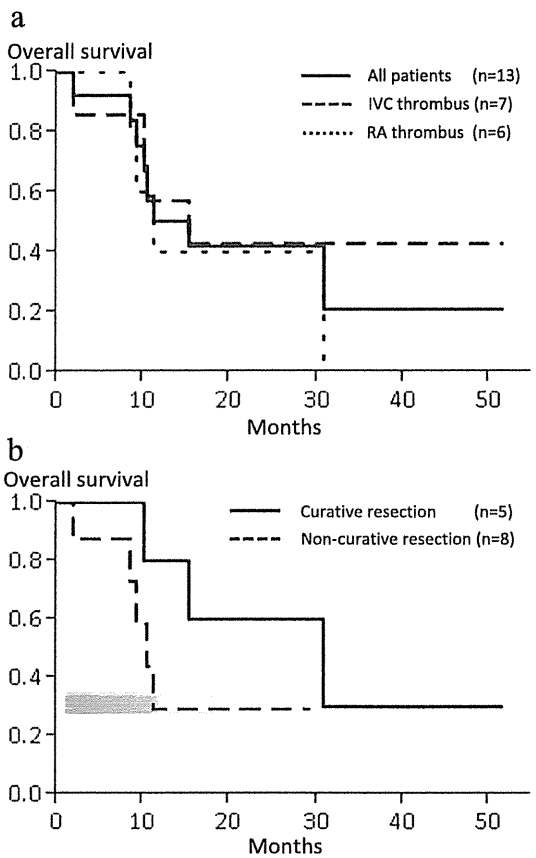

Figure 2 Survival curve for patients with hepatocellular carcinoma (HCC) and inferior vena cava (IVC) and right atrium (RA) thrombi. (a) Overall survival curve for all patients (n = 13), patients with IVC thrombus, and patients with RA thrombus. $P = 0.6056$ by log-rank test, IVC thrombus versus RA thrombus. **(b)** Overall survival curve for patients who underwent curative surgical resection and noncurative resection. $P = 0.2103$ by log-rank test, curative resection versus noncurative resection.

IVC or RA thrombectomy, because the degree of bleeding is a major predictive factor for operative morbidity and mortality [21]. In this study, hepatic parenchymal transection was routinely performed prior to thrombectomy using the Pringle maneuver. IVC occlusion at the suprahepatic portion with bulky tumor thrombi evokes Budd-Chiari syndrome and massive hepatic congestion [28]. In this study, we observed hepatic congestion in patients with outflow obstruction of spared hepatic veins by a massive tumor thrombus. Furthermore, occlusion of two of three major hepatic veins by venous invasion induced hepatic congestion, even though the spared hepatic vein was not obstructed. We used extracorporeal bypass from the PV and IVC to SVC to decompress the liver parenchyma and decrease bleeding during hepatic transection in two patients with an RA thrombus [29]. We performed IVC thrombectomy under a favorable field with good bleeding control by HVE. The duration of HVE, which could trigger hemodynamic deterioration, was short enough. Although CPB was mandatory for RA thrombectomy and was accompanied with a larger amount of blood loss and a higher rate of blood transfusion, patients with RA thrombi required minimal ICU stays and shorter postoperative hospitalization. These procedures contributed to a low incidence of postoperative non-serious complications that were medically manageable. Furthermore, we did not observe any operative mortality in this study. Therefore, hepatectomy with IVC or RA thrombectomy, although technically challenging, can be performed safely with appropriate inflow vascular control for patients with good hepatic reserve. Because almost all thrombi had capsules and did not adhere to the wall of the IVC or RA, they were simply removed by thrombectomy without wall resection. Although some authors indicate the efficacy of IVC resection, the benefits are controversial [30]. We experienced two cases of intra-IVC recurrence after surgery (Table 5); therefore, the management of such tumor thrombi should be reconsidered.

The prognosis of HCC patients with IVC or RA tumor thrombi is extremely poor. Earlier observations revealed a median survival duration after diagnosis of 1 to 5 months for untreated patients [4,15,31]. Although there is no consensus on the therapeutic options for HCC with IVC or RA thrombi, nonsurgical treatments such as TACE, as well as radiotherapy and chemotherapy, have been attempted. Previous reports concerning the therapeutic benefits of TACE with or without radiotherapy revealed insufficient results, with a median survival duration of 9.2 months (range, 4.2 to 18.4 months) [4,8,11-13,32]. Currently, the outcome of systemic chemotherapy for HCC has been disappointing, although sorafenib, which is the only effective agent against HCC, demonstrated a slightly better prognosis of 10.7 months in patients with unresectable HCC [33]. Some case reports have suggested the efficacy of surgical resection, and, recently, Wang *et al.* reported the significant superiority of a surgical approach to HCC with IVC or RA thrombi, with a median survival duration of 19 months, compared with TACE with or without chemoradiotherapy or no treatment [4,6,9,16, 17,20,26,27,34]. This study included 56 patients, of whom 25 underwent surgery, although 7 had an RA thrombus and only 3 underwent surgical resection for the same [4]. Therefore, the therapeutic benefit of surgical resection for HCC with an RA thrombus remains unclear. In the present study, the median overall survival duration of patients with an IVC or RA thrombus was comparable at 15.3 months and 11.2 months, respectively (Figure 2a). This finding indicates the equivalent therapeutic efficacy of surgical resection for RA or IVC thrombi. These results for patient survival are slightly worse than those of the

previous study [4] because our study included patients who underwent non-curative resection. The median survival duration of patients who underwent curative resection was 30.8 months, which is longer than that in the previous report [4] (Figure 2b). This result also surpasses nonsurgical treatment with sorafenib, which resulted in median survival duration of 8.1 months in patients with macrovascular invasion [35].

All patients who underwent curative surgical resection experienced local recurrence or distant metastasis in the early postoperative phase, despite the fact that almost all patients received adjuvant chemotherapy (Table 5). It has been recognized that the poor prognosis of HCC with tumor thrombi in the IVC or RA is strongly related to a high incidence of postoperative recurrence at a relatively early stage, even after curative surgery. In this study, most patients who underwent curative resection developed postoperative lung metastases and intrahepatic recurrence at an equal rate. Preoperative or intraoperative dissemination of tumor cells to the lung can contribute to postoperative metastatic recurrence. To prevent potential intraoperative dissemination by intraoperative handling, some authors indicated the benefit of separated thrombectomy before hepatic transection [36]. However, it could be technically difficult to remove a thrombus en-bloc without up-front hepatic transection; therefore, further improvements in surgical techniques are required. To date, there is no clear modality established for preventing HCC recurrence [4,16,37]. The efficacy of preoperative radiotherapy is indicated for PV tumor thrombi [38]; however, the benefit for IVC or RA thrombi is unclear and there remains a risk of thrombi dislodgment during radiation. In this study, locally recurrent tumors were controlled by TACE or RFA and distant metastatic tumors were treated by chemotherapy with or without radiotherapy or surgical excision if resectable. These vigorous repetitive treatments contribute to improvement in survival, even after recurrence.

Surgical resection is commonly contraindicated for patients with unresectable metastatic tumors because incomplete resection is a crucial factor for poor prognosis [6]. However, hepatic lesions, but not distant metastasis, are the major factors influencing poor prognosis for death in the early postoperative phase [39]. On the basis of the fact that the survival duration of patients with IVC or RA thrombi is extremely short with nonsurgical treatment, distant metastasis itself should not be considered a contraindication for surgery. In this study, eight patients underwent noncurative surgery, and the median survival duration of 10.5 months was relatively better than that for patients who underwent nonsurgical treatment or no treatment in previous studies, including patients who received sorafenib, which is the only highly evidenced agent for advanced HCC treatment and results in median survival duration of 8.9 months in patients with extrahepatic metastases [4,8,11-13,15, 30-32,35]. Recent reports indicate the efficacy of aggressive treatment for HCC metastases, including those to the lung, adrenal gland, and lymph nodes, by surgery or radiotherapy [40-42]. In this study, a patient with lung metastases at primary surgery underwent resection of the metastases and survived for 29.3 months. These findings indicate that reductive surgical resection can be justified in patients with IVC or RA thrombi accompanied by distant metastases or unresectable intrahepatic metastases. Control of the life-threatening progression of intrahepatic HCC and prevention of unexpected death by pulmonary embolism would give these patients a chance to undergo multidisciplinary treatments for improving survival. Therefore, if intrahepatic HCC and IVC or RA thrombi can be totally or partially resected, surgical resection may be beneficial.

The limitations of this study include its retrospective design, its single-center design, the small sample size, and patient heterogeneity. Because IVC and RA thrombi associated with HCC are rare, a multicenter prospective study with a large patients sample is necessary to definitively establish the benefits of surgical management.

Conclusions

In conclusion, surgical resection of HCC with IVC or RA thrombosis can be performed safely with appropriate inflow vascular control in patients with good hepatic reserve. We suggest that aggressive surgical resection may be more beneficial than existing therapeutic modalities; however, early recurrence and treatment of recurrent or metastatic tumors remain unresolved issues. Further studies on adjuvant therapies and establishment of therapeutic strategies for recurrent and metastatic tumors are important challenges to improve survival.

Abbreviations

Af: Atrial fibrillation; ARF: Acute renal failure; HBV: Hepatitis B virus; CDDP: Cisplatin; CPB: Cardiopulmonary bypass; CT: Computed tomography; 5-FU: 5-fluorouracil; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HVE: Hepatic vascular exclusion; ICG R_{15}: Indocyanine green retention rate at 15 minutes after injection; IRHV: Inferior right hepatic vein; IVC: Inferior vena cava; LHV: Left hepatic vein; MHV: Middle hepatic vein; MRI: Magnetic resonance imaging; PV: Portal vein; RA: Right atrium; RAdV: Right adrenal vein; RFA: Radio frequency ablation; RHV: Right hepatic vein; S-1: Tegafur gimeracil oteracil potassium; SVC: Superior vena cava; TACE: Transarterial chemoembolization; UFT: Tegafur uracil.

Competing interests

The authors have no conflicts of interest to declare.

Authors' contributions

KW and TK designed the research, KW, TK, HY, TK, HK, YT, KN, TS, ST, and AT contributed to acquisition of data, and KW and TK analyzed and interpreted data. All authors read and approved the final manuscript.

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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

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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

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

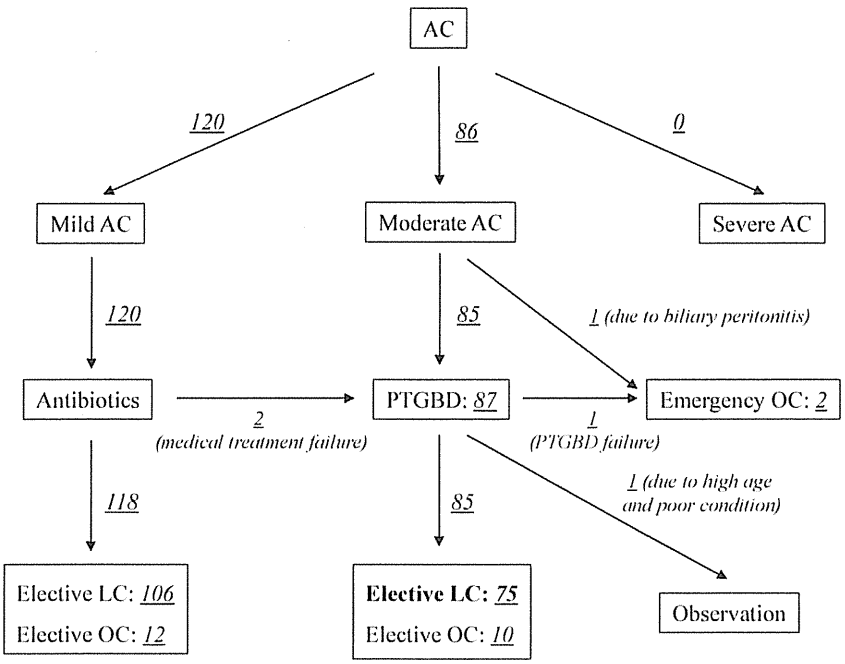

Fig. 1 The flow chart of treatment for acute cholecystitis (AC) according to the strategy in our institute. In our institute, early cholecystectomy including laparoscopic cholecystectomy (LC) and open cholecystectomy (OC) is not adopted. Thus, elective cholecystectomy after antibiotics therapy was performed 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

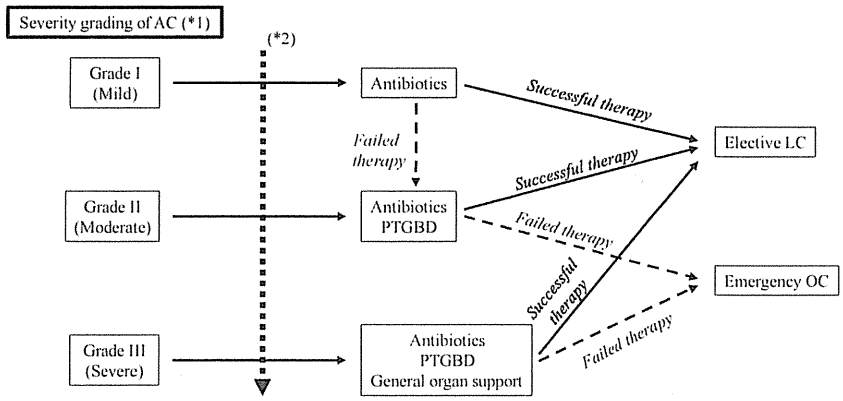

Fig. 2 The treatment strategy for acute cholecystitis (AC) in patients under antithrombotic therapy in our institute. (*1): Severity grading of AC is based on Tokyo guideline 2013. (*2): Antithrombotic drugs are immediately discontinued and heparin replacement is considered if needed. *PTGBD* percutaneous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

To the Editor: The incidence of liver cirrhosis after choledochal cyst (CC) has been reported to be 2.1% to 11.8% (1). It is particularly important to prevent liver damage progressing toward cirrhosis in CC. It is unknown whether hepatic fibrosis in symptomatic patients after birth with prenatally diagnosed CC is present or absent, especially regarding the severity of hepatic fibrosis.

Of the 27 cases with CC, 8 were diagnosed prenatally (mean fetal age, 27 weeks [20–36 weeks]), and were categorized into 2 groups: a symptomatic infant group including 5 patients (mean fetal age, 27 weeks [20–31 weeks] and 10-year follow-up] and an asymptomatic infant group including 3 patients (mean fetal age, 28 weeks [20–36 weeks] and 4-year follow-up).

Histological findings of the hematoxylin and eosin–stained liver biopsy specimens, especially with regard to the developmental degree of liver fibrosis, were classified into 5 grades (Ohkuma classification) (2). The symptomatic CC group consisted of 1 case of grade 0 and 4 cases of grade 1. The asymptomatic CC group consisted of 1 case of grade 0 and 2 cases of grade 1. There was a histological difference between symptomatic and asymptomatic infants with prenatally diagnosed CC ($P = 0.0312$).

It is important to keep in mind that liver fibrosis is significantly positive in symptomatic infants with prenatally diagnosed CC, although it is mild, compared with that in asymptomatic infants. The conclusion drawn from this study is consistent with the hypothesis that timely surgical intervention can lead to the reversal of liver fibrosis.

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Colon Preparation for Children: The Quest for the Ideal Protocol

To the Editor: The publication of cleansing colonic protocols for colonoscopy procedure in children in the February 2013 issue of *JPGN* was a wake-up call to address this topic (1–4). These prospective studies confirmed the acceptability and safety of polyethylene glycol (PEG) 3350, but its low effectiveness (adequate preparation <90%) showed that we are yet a long way from the optimal protocol needed for our patients.

PEG 3350 has been proven to be safe, palatable, acceptable, and successful for constipation, fecal impaction, and colon cleansing protocols. It is time to conclude that PEG 3350 is the best solution we have for colon cleansing in children. Unfortunately, to date, there is no criterion standard protocol for colon cleansing, and each medical center uses its "in house" protocol, in many cases without carefully documenting the end result. Patel and Pashankar (4) stated that there are few head-to-head comparisons between protocols in children and even fewer comparisons between the PEG 3350 solutions, adjusting for timing, duration, dosing, and so on. To find the PEG 3350 criterion standard protocol for children, studies adjusting these variables and measuring outcomes need to be conducted. The various attempts to shorten the protocol, almost being absurd (even counting the hours!), are usually blocked by the limitation of human consumption, and is the wrong direction to follow. We recently completed the first head-to-head comparison between 2 PEG 3350–based protocols (5,6) to find the superior protocol and to examine protocol reproducibility (7). In the quest for the criterion standard, we need to concentrate on the simplicity, acceptability, reliability of grading, and, most important, the rate of successful preparation. So far, we are a long way from an adequate success rate.

In summary, the quest to find the best colon cleansing protocol for children needs to be pursued with a change of direction. I believe PEG 3350 should be the solution used in all protocols, with the aim of finding the best variables associated with those protocols. In addition, we should always apply head-to-head comparison between protocols. Only in this way will we be able to assess reproducibility and superiority of one protocol against the other. Without it, we will fail in our mission to provide the best available intervention for our children.

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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.

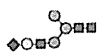
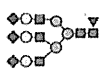
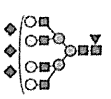
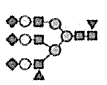
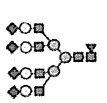
N-Glycan Purification and Modification by Glycoblotting. Glycoblotting of sample mixtures containing whole serum *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (500 μL) (10 mg/ml suspension; Sumitomo Bakelite) were aliquoted into 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

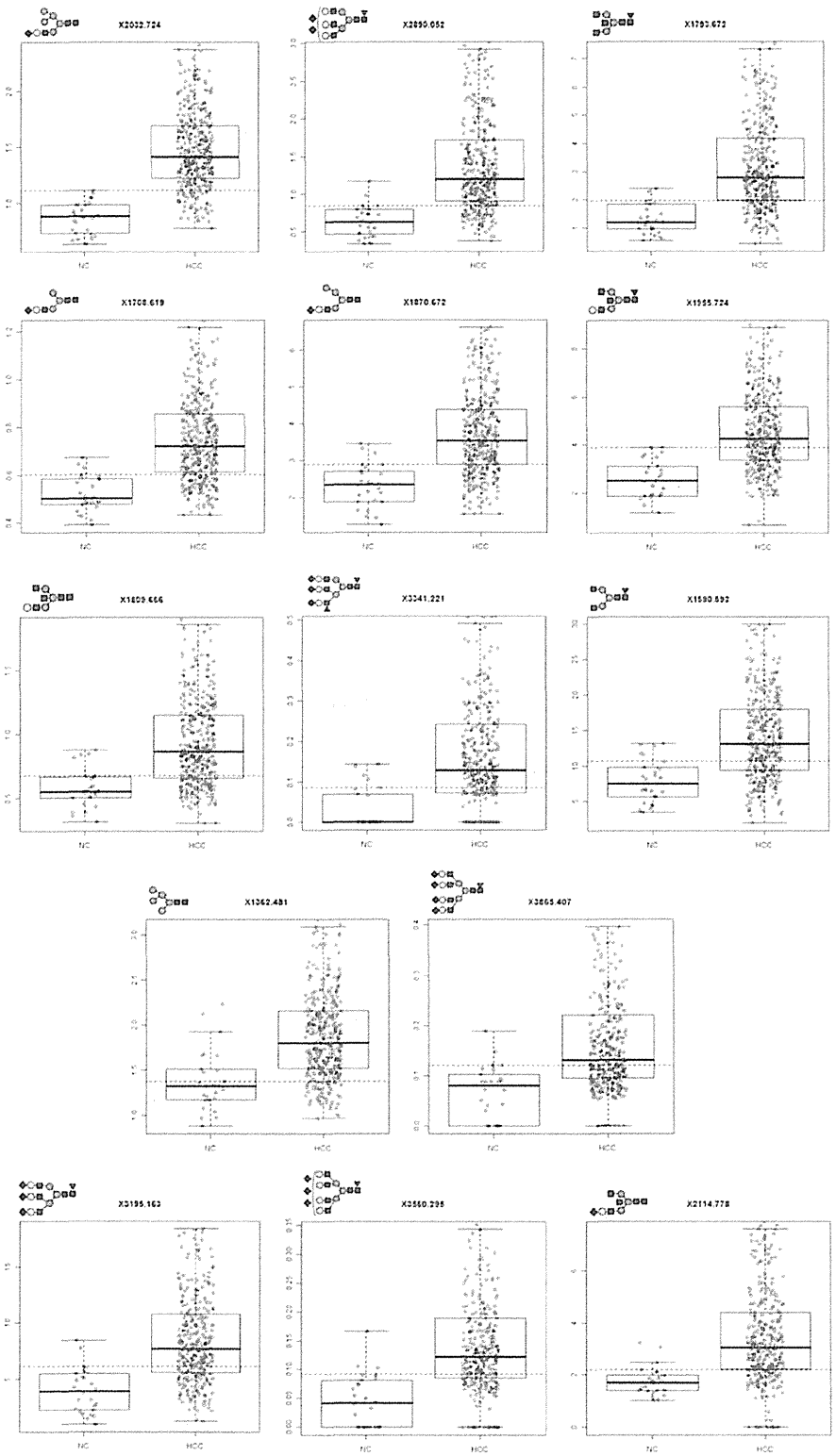


Fig. 1. Boxplots of the disease-free individuals (NC) and HCC patients for the selected 14 *N*-glycans. The dotted lines in the graphs represent the cutoff values determined in this analysis. These graphs were drawn using R v. 2.12.1.