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

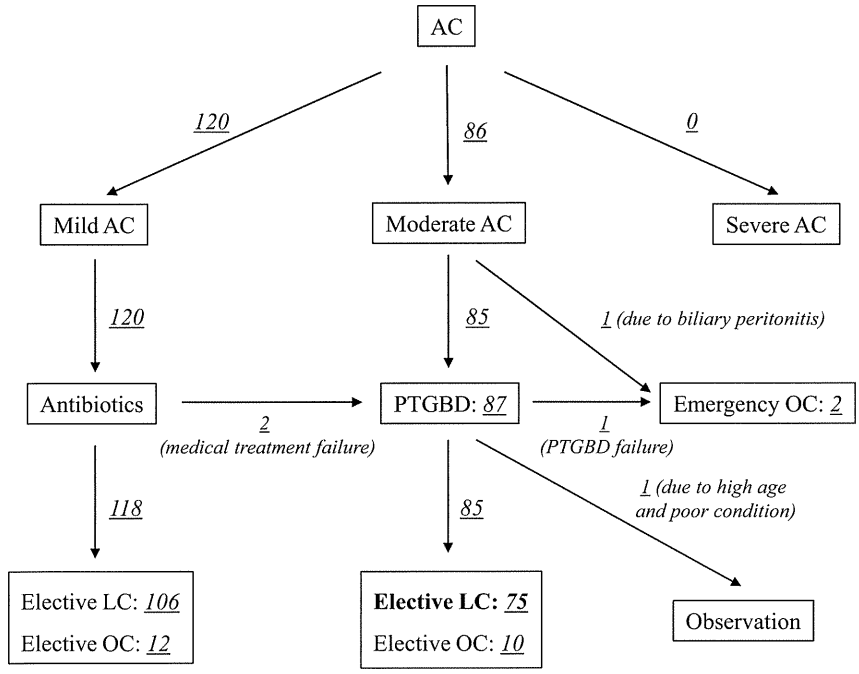

Fig. 1 The flow chart of treatment for acute cholecystitis (AC) according to the strategy in our institute. In our institute, early cholecystectomy including laparoscopic cholecystectomy (LC) and open cholecystectomy (OC) is not adopted. Thus, elective cholecystectomy after antibiotics therapy was performed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

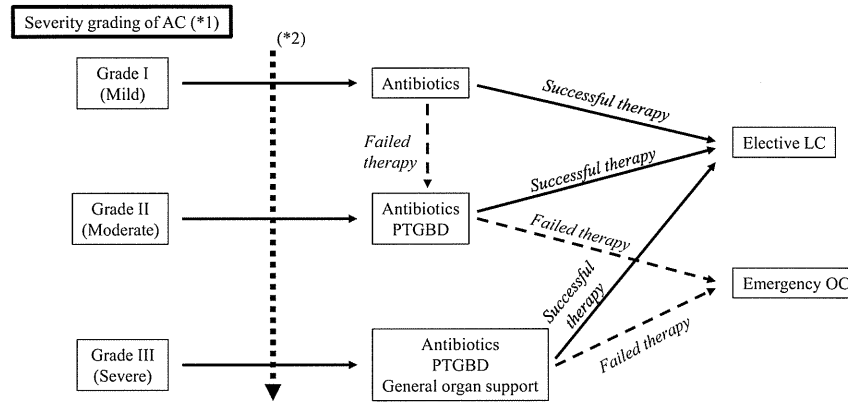

Fig. 2 The treatment strategy for acute cholecystitis (AC) in patients under antithrombotic therapy in our institute. (*1): Severity grading of AC is based on Tokyo guideline 2013. (*2): Antithrombotic drugs are immediately discontinued and heparin replacement is considered if needed. *PTGBD* percutaneous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

Surgical management of hepatocellular carcinoma with tumor thrombi in the inferior vena cava or right atrium

Kenji Wakayama†, Toshiya Kamiyama*†, Hideki Yokoo†, Tatsuhiko Kakisaka†, Hirofumi Kamachi†, Yosuke Tsuruga†, Kazuaki Nakanishi†, Tsuyoshi Shimamura†, Satoru Todo† and Akinobu Taketomi†

Abstract

Background: The prognosis for advanced hepatocellular carcinoma (HCC) with tumor thrombi in the inferior vena cava (IVC) or right atrium (RA) is poor, and there is no established effective treatment for this condition. Thus study aimed to evaluate the efficacy of surgical resection and prognosis after surgery for such cases.

Methods: Between January 1990 and December 2012, 891 patients underwent hepatectomy for HCC at our institution. Of these, 13 patients (1.5%) diagnosed with advanced HCC with tumor thrombi in the IVC or RA underwent hepatectomy and thrombectomy. Data detailing the surgical outcome were evaluated and recurrence-free and overall survival rates were calculated using the Kaplan-Meier method.

Results: Seven patients had an IVC thrombus and six had an RA thrombus. Extra-hepatic metastasis was diagnosed in 8 of 13 patients. Surgical procedures included three extended right lobectomies, three extended left lobectomies, five right lobectomies, and two sectionectomies. Right adrenal gland metastases were excised simultaneously in two patients. All IVC thrombi were removed under hepatic vascular exclusion and all RA thrombi were removed under cardiopulmonary bypass (CPB). Four patients (30.8%) experienced controllable postoperative complications, and there was no surgical mortality. The mean postoperative hospital stay for patients with IVC and RA thrombi was 23.6 ± 12.5 days and 21.2 ± 4.6 days, respectively. Curative resection was performed in 5 of 13 cases. The 1- and 3-year overall survival rates were 50.4%, and 21.0%, respectively, and the median survival duration was 15.3 months. The 1- and 3-year overall survival rates for patients who underwent curative surgical resection were 80.0% and 30.0%, respectively, with a median survival duration of 30.8 months. All patients who underwent curative resection developed postoperative recurrences, with a median recurrence-free survival duration of 3.8 months. The 1-year survival rate for patients who underwent noncurative surgery and had residual tumors was 29.2%, with a median survival duration of 10.5 months.

Conclusions: Aggressive surgical resection for HCC with tumor thrombi in the IVC or RA can be performed safely and may improve the prognoses of these patients. However, early recurrence and treatment for recurrent or metastatic tumors remain unresolved issues.

Keywords: Hepatocellular carcinoma, Inferior vena cava, Right atrium, Tumor thrombus, Surgery

* Correspondence: t-kamiya@med.hokudai.ac.jp
†Equal contributors
Department of Gastroenterological Surgery 1, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo, Japan

Background

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a propensity for invading intrahepatic blood vessels such as the portal vein (PV) or hepatic vein in advanced stages [1]. Further extension of tumor thrombi from any of the three main hepatic veins or the right inferior hepatic vein can give rise to thrombi in the inferior vena cava (IVC) or right atrium (RA) [1-3]. Commonly, the prognosis of HCC patients presenting with IVC or RA thrombosis is extremely poor [4-6], and there is no established management for such cases [4,5,7-17]. Surgical removal of IVC and RA thrombi combined with hepatectomy is the only radical treatment to decrease the risk of systemic metastasis and sudden death due to pulmonary embolism or occlusion of the tricuspid valve with a tumor thrombus [18-20]. However, aggressive surgical resection is not common because the surgical approach to IVC and RA thrombi is considered complicated and hazardous and is applicable only in limited cases with good hepatic reserve [4,6,9,16,17]. Therefore, the efficacy of surgical treatment for HCC with IVC or RA thrombi remains unclear. In this study, we retrospectively investigated the surgical outcomes and prognoses of patients who underwent surgery for HCC with IVC or RA tumor thrombi in a single institution to clarify the safety and efficacy of surgical resection.

Methods

Patients and diagnoses

Between January 1990 and December 2012, 891 patients underwent hepatectomy for HCC at the Department of Gastroenterological Surgery, Hokkaido University, Japan. The diagnosis of HCC was determined by enhanced computed tomography (CT) and magnetic resonance imaging (MRI). IVC or RA thrombi were evaluated by CT (Figure 1). Among those studied, 13 patients (1.5%) diagnosed with advanced HCC and tumor thrombi in the IVC or RA underwent hepatectomy. This study was approved by the Institutional Review Board of the Hokkaido University School of Advanced Medicine.

The mean age at diagnosis was 63.4 years. The most common cause of HCC was hepatitis B virus (HBV) infection (53.8%), followed by hepatitis C virus (HCV) infection (15.4%). A total of 12 (92.3%) patients were male, and according to the Child-Pugh classification, all cases had Child-Pugh class-A disease. Six (46.2%) patients had a single tumor and seven (53.8%) had multiple tumors. The mean main tumor size was 11.8 cm, with nine tumors (69.2%) located in the right lobe and four (30.8%) in the left lobe. Extra-hepatic metastases were detected in eight of thirteen (61.5%) patients (five with lung, two with right adrenal gland and one with mediastinal lymph node metastases). Seven (53.8%) patients had an IVC thrombus and five (46.2%) had an RA thrombus (Table 1).

The tumor thrombus arose from the right hepatic vein in four patients (30.8%), middle hepatic vein in three (23.1%), left hepatic vein in one (7.7%), inferior right hepatic vein in two (15.4%), right hepatic vein combined with the middle hepatic vein in one (7.7%), and right hepatic vein combined with the inferior right hepatic vein in one (7.7%). In one patient with right adrenal gland metastasis, the tumor thrombus arose from the right adrenal vein (7.7%). Two patients had a mural thrombus and eleven had a massive thrombus. The massive thrombi in 10 patients did not completely occlude the IVC because circinate or arc-like luminal flow in the IVC around the tumor thrombi was present and the outflow canals of the intact hepatic veins were maintained. The tumor thrombus of one patient completely occluded the IVC inferior to the influx of hepatic veins accompanied by an aggregating blood thrombus. The outflow canals of the intact hepatic veins were severely narrowed but not completely occluded. One patient with a thrombus completely occluding the IVC and two patients with a massive IVC thrombus suffered preoperative renal insufficiency, and two had evident leg edema (Table 2).

Surgical procedures

A lobectomy was performed for patients with an indocyanine green retention rate at 15 minutes after injection (ICG R_{15}) of <15% and total bilirubin levels of <1.5 mg/dl without ascites. Patients with an ICG R_{15} of 15 to 20% and total bilirubin levels of 1.5 to 2.0 mg/dl were eligible for sectionectomy according to our criteria [21]. The type of surgical procedure was selected based on Couinaud's classification [22] and included three extended right hepatectomies, three extended left hepatectomies, five right hepatectomies, and two sectionectomies accompanied by thrombectomies. The right adrenal gland was resected simultaneously in two patients with right adrenal gland metastases (Table 3).

Hepatic resections were performed with an ultrasonic dissector using the Pringle maneuver in all cases. All IVC thrombi were removed under hepatic vascular exclusion (HVE). Before thrombectomy, hepatic transection was performed and the IVC was clamped below and above the liver. The IVC thrombus was excised en-bloc from the incised IVC, with satisfactory visualization of the intraluminal space under HVE. The IVC incision was closed by a simple continuous suture without a patch. All RA thrombi were removed under cardiopulmonary bypass (CPB). Following a laparotomy, a median sternotomy was performed to prepare for prompt CPB in anticipation of an undesirable pulmonary tumor embolism from a dislodged thrombus. Hepatic transection was then performed. The liver was handled gently, particularly if the thrombus had a long, thin neck, to

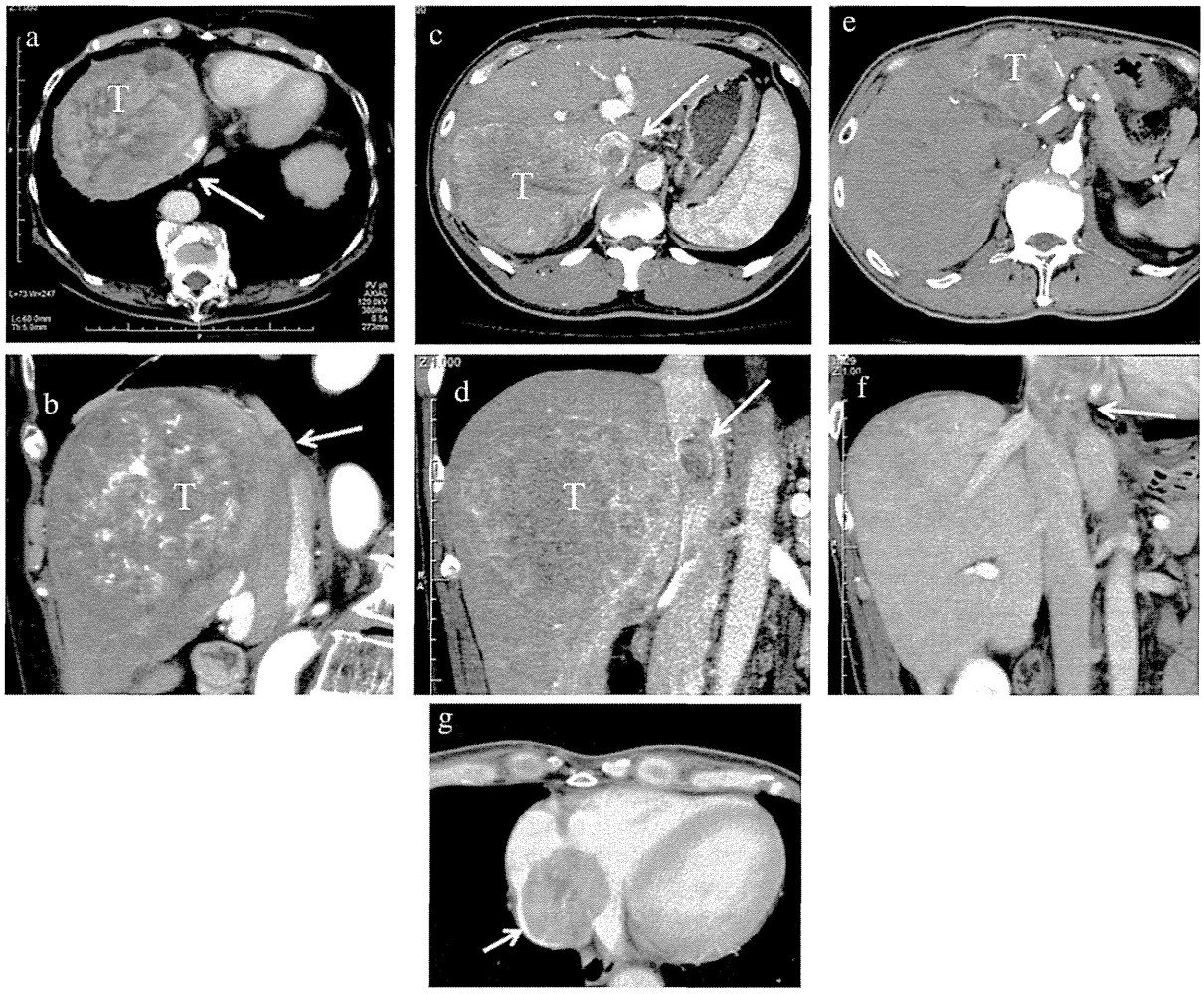

Figure 1 Representative computed tomography findings of hepatocellular carcinoma (HCC) with inferior vena cava (IVC) and right atrium (RA) thrombi. (**a** and **b**) A large HCC lesion in the right lobe (T), with a tumor thrombus arising from the right hepatic vein into the IVC (arrow). (**c** and **d**) HCC in the right lobe (T), with a tumor thrombus arising from the inferior right hepatic vein into the IVC (arrow). (**e**, **f**, and **g**) HCC in the left lobe (T), with a tumor thrombus arising from the left hepatic vein into the RA (arrow).

prevent dissemination of tumor thrombi. Then, the superior vena cava (SVC) and IVC below the liver were clamped and blood flow was bypassed to the ascending aorta via an oxygenator. The RA was incised and the thrombus was excised en-bloc under direct vision. In most cases, the RA was reconstructed by simple sutures, but in two cases, an invaded RA wall was partially excised and reconstructed using an artificial graft or pericardial patch. In addition to CPB, one patient with complete IVC occlusion accompanied by severe obstruction of intact hepatic outflow and one patient with tumor thrombi that arose from two major hepatic veins showed gross hepatic congestion due to outflow block at surgery. These cases needed extracorporeal bypass from the portal vein (PV) and IVC to SVC (Table 3). In all cases, thrombi were intraoperatively monitored by transesophageal echocardiography. In this study, we defined curative resection as macroscopic complete excision of the tumors, including metastatic lesions.

Follow up

The median duration of follow up was 11.2 (range, 1.8 to 51.8) months. Hospital death was defined as death occurring within 30 days of the first hospitalization. After surgery, CT or MRI was performed at 1- to 3-month intervals to determine recurrence. Data on surgical outcomes, postoperative management, recurrence, treatment of recurrence, and survival was analyzed for all cases.

Statistical analysis

Survival rates were analyzed by the Kaplan-Meier method and statistical significance was determined by

Table 1 Characteristics of patients and tumors

Characteristic	Value
Total number of patients	13
Age, years	
Mean ± SD (range)	63.4 ± 11.8 (37 to 86)
Sex	
Male/female	12/1
Hepatitis B virus	
Positive/negative	7/6
Hepatitis C virus	
Positive/negative	2/11
Child-Pugh classification	
A/B/C	13/0/0
Main tumor location	
Anterior/posterior/median/lateral section	5/4/3/1
Tumor size, cm	
Mean ± SD (range)	11.8 ± 4.3 (3.5–19)
Number of tumors	
Single/multiple	6/7
Extension of thrombus	
Inferior vena cava/right atrium	7/6
Preoperative extrahepatic metastases	
None/lung/adrenal gland/lymph nodes	5/5/2/1
Status of metastases after surgery	
Resected/regressed or stationary/progressed	3/1/4
Postoperative metastatic recurrence	
Liver/lung/lymph node/adrenal gland/inferior vena cava/brain	8/7/4/2/2/2

the log-rank test using JMP Pro 10.0.0 software (SAS, Cary, NC, USA). Significance was defined as $P < 0.05$.

Results

Surgical outcomes and postoperative complications

With regard to patients with an IVC thrombus, the mean surgical duration was 349 ± 30 minutes, the median blood loss was 950 ± 100 ml, and the mean HVE duration was 8.8 ± 3.1 minutes. Two of seven (28.6%) patients needed blood transfusions. No patient required an ICU stay, and the mean postoperative hospital stay was 23.6 ± 12.5 days. After surgery, one patient experienced biloma and one experienced controllable ascites. With regard to patients with an RA thrombus, the mean surgical duration was 608 ± 169 minutes, the median blood loss was 6540 ± 5404 ml, and the mean CPB duration was 32.2 ± 18.3 minutes. Five of six (83.3%) patients needed blood transfusions. The mean postoperative ICU stay was 1.7 ± 0.8 days and the mean postoperative hospital stay was 21.2 ± 4.6 days. After surgery, one patient experienced acute renal failure and one experienced atrial fibrillation, but these patients recovered with medical therapy. There was no postoperative mortality. All IVC and RA thrombi were excised completely. Curative resection was performed in five of thirteen (38.5%) cases (Table 4).

Postoperative management

Among the five patients (38.5%) who underwent curative resection, adjuvant systemic chemotherapy was administered to four. The chemotherapeutic agents used in combination included intravenous 5-fluorouracil (5-FU; 500 mg weekly) and peroral tegafur uracil (UFT; 300 mg daily) in three patients and peroral UFT (300 mg daily) in one. One patient was followed up without adjuvant chemotherapy.

Tumors remained after surgery in eight (61.5%) patients, including lung metastases in four, intrahepatic metastases in two, both intrahepatic and lung metastases in one, and mediastinal lymph node metastases in one. Residual lung metastases were treated with oral administration of UFT in two patients, 5-FU + UFT in one patient, and oral administration of tegafur gimeracil oteracil potassium (S-1) followed by surgical resection in one patient. Unresectable intrahepatic metastases were treated with UFT in two patients and transarterial chemoembolization (TACE) in one patient. A patient with residual mediastinal lymph node metastasis received radiation after surgery.

Recurrence and survival

All five patients who underwent curative resection experienced postoperative recurrences. Intrahepatic recurrences appeared in all five patients, lung metastases in four, intra-IVC metastases in one, and left adrenal gland metastases in one patient. The median recurrence-free survival duration of the patients who underwent curative resection was 3.8 months. Intrahepatic recurrences were treated with TACE in three patients, radiofrequency ablation (RFA) in two, and radiotherapy in one patient. Lung metastases were treated with systemic chemotherapy in three patients (5-FU + UFT in two, cisplatin (CDDP) + S-1 followed by oral administration of sorafenib in one patient), and surgical resection in one patient. Left adrenal gland metastases were surgically excised.

Among the eight patients who underwent noncurative resection, four of five with lung metastases exhibited progression of the metastases. In one patient, lung metastasis was resected but recurred after resection. Intrahepatic residual tumors in three patients progressed after surgery; however, mediastinal lymph node metastases treated by irradiation remained unchanged. Among these eight patients, seven experienced further dissemination of the tumor to new locations, including the lung in three, lymph

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.

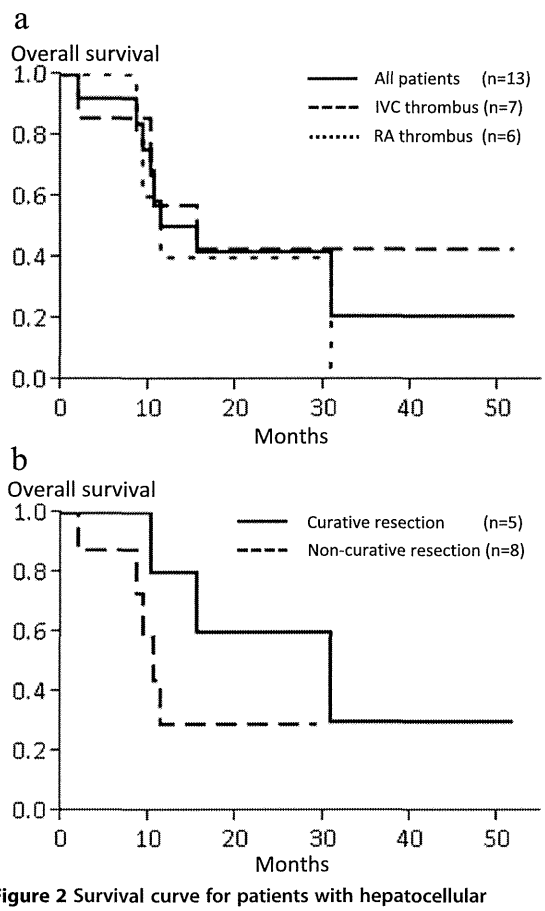

Figure 2 Survival curve for patients with hepatocellular carcinoma (HCC) and inferior vena cava (IVC) and right atrium (RA) thrombi. (a) Overall survival curve for all patients (n = 13), patients with IVC thrombus, and patients with RA thrombus. $P = 0.6056$ by log-rank test, IVC thrombus versus RA thrombus. **(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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Heat shock factor 1 accelerates hepatocellular carcinoma development by activating nuclear factor-κB/mitogen-activated protein kinase

Makoto Chuma*, Naoya Sakamoto, Akira Nakai[1], Shuhei Hige, Mitsuru Nakanishi, Mitsuteru Natsuizaka, Goki Suda, Takuya Sho, Kanako Hatanaka[2], Yoshihiro Matsuno[2], Hideki Yokoo[3], Toshiya Kamiyama[3], Akinobu Taketomi[3], Gen Fujii[4], Kosuke Tashiro[5], Yoko Hikiba[6], Mitsuaki Fujimoto[1], Masahiro Asaka and Shin Maeda[7]

Department of Gastroenterology and Hepatology, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan, [1]Department of Biochemistry and Molecular Biology, Yamaguchi University, Ube, Japan, [2]Department of Pathology and [3]Department of Gastroenterological Surgery I, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan, [4]Division of Cancer Prevention, National Cancer Center Research Institute, Tokyo, Japan, [5]Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka, Japan, [6]Division of Gastroenterology, Institute for Adult Diseases, Asahi Life Foundation, Tokyo, Japan and [7]Department of Gastroenterology, Yokohama City University, Yokohama, Japan

*To whom correspondence should be addressed. Tel: +81 11-716-1611; Fax: +81 11-706-7867;
Email: mchuuma@med.hokudai.ac.jp

Heat shock factor 1 (HSF1), a major transactivator of stress responses, has been implicated in carcinogenesis in various organs. However, little is known about the biological functions of HSF1 in the development of hepatocellular carcinoma (HCC). To clarify the functional role of HSF1 in HCC, we established HSF1-knockdown (HSF1 KD) KYN2 HCC cells by stably expressing either small hairpin RNA (shRNA) against HSF1 (i.e. HSF1 KD) or control shRNA (HSF1 control). Tumorigenicity was significantly reduced in orthotopic mice with HSF1 KD cells compared with those with HSF1 control cells. Reduced tumorigenesis in HSF1 KD cells appeared attributable to increased apoptosis and decreased proliferation. Tumor necrosis factor-α-induced apoptosis was increased in HSF1 KD cells and HSF1$^{-/-}$ mouse hepatocytes compared with controls. Decreased expression of IκB kinase γ, a positive regulator of nuclear factor-κB, was also observed in HSF1 KD cells and HSF1$^{-/-}$ mouse hepatocytes. Furthermore, expression of bcl-2-associated athanogene domain 3 (BAG3) was dramatically reduced in HSF1 KD cells and HSF1$^{-/-}$ mouse hepatocytes. We also found that epidermal growth factor-stimulated mitogen-activated protein kinase signaling was impaired in HSF1 KD cells. Clinicopathological analysis demonstrated frequent overexpression of HSF1 in human HCCs. Significant correlations between HSF1 and BAG3 protein levels and prognosis were also observed. In summary, these results identify a mechanistic link between HSF1 and liver tumorigenesis and may provide as a potential molecular target for the development of anti-HCC therapies.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the third leading cause of cancer death worldwide (1). Despite marked advances in diagnostic and therapeutic techniques, prognosis remains unsatisfactory for HCC patients (2,3). An understanding of HCC carcinogenesis at the molecular level is thus urgently needed in order to identify novel molecular targets for the development of more effective therapies.

Heat shock factor 1 (HSF1) is the main regulator of the heat shock response, which is involved in protecting cells and organisms from heat, ischemia, inflammation, oxidative stress and other noxious conditions (4,5). Under various forms of physiological stress, HSF1 drives the production of heat shock proteins (HSPs), such as HSP27, HSP70 and HSP90, which act as protein chaperones (5,6). The functions of HSF1 are not limited to increasing the expression of chaperones; HSF1 also modulates the expression of hundreds of genes other than chaperones that are critical for survival under an array of potentially lethal stressors (6–8). As a result, HSF1 influences fundamental cellular processes such as cell cycle control, protein translation, glucose metabolism and proliferation (7–12). In human tumors, constitutive expression of Hsp27, Hsp70 and Hsp90 at high levels predicts poor prognosis and resistance to therapy (13–15). These effects are often attributable to HSF1-dependent mechanisms (16). Thus, as a master regulator of cellular processes, the roles of HSF1 in carcinogenesis and tumor progression are now emerging. Several recent investigations using mouse models have suggested that HSF1 is involved in carcinogenesis (9,17). In clinical samples, HSF1 is often constitutively expressed at high levels in a variety of tumors, including breast cancer (7,18), pancreatic cancer (19), prostate carcinoma (20) and oral squamous cell carcinoma (21).

Hepatocarcinogenesis is a multistep process, in the majority of cases slowly developing within a well-defined etiology of viral infection and chronic alcohol abuse, leading to the chronic hepatitis and cirrhosis that are regarded as preneoplastic stages (22). A great number of factors, receptors and downstream elements of signaling cascades regulate proliferation and apoptosis. Dysregulation of the balance between cell proliferation and apoptosis thus plays a critical role in hepatocarcinogenesis (23,24). Two of the major pathways of cell proliferation and apoptosis are nuclear factor kappa B (NF-κB) signaling and mitogen-activated protein kinase (MAPK) signaling. NF-κB transcription factors are critical regulators of genes involved in inflammation and the suppression of apoptosis. NF-κB has been shown to be instrumental for tumor promotion in colitis-associated cancer and inflammation-associated liver cancer (25,26). Activation of the extracellular signal-regulated kinase (ERK)/MAPK pathway regulates many important cellular processes, such as proliferation, differentiation, angiogenesis, survival and cell adhesion (27). Importantly, the ERK/MAPK pathway is constitutively activated in HCC (28).

The present study investigated the biological influences of HSF1 in HCC cell proliferation and apoptosis involving the NF-κB and MAPK signal pathways. We found that HSF1 deficiency significantly diminished NF-κB and MAPK activation in primary hepatocytes and HCC cells, so HSF1 deficiency inhibited the development of HCC. Furthermore, clinicopathological analysis demonstrated a significant correlation between HSF1 protein level and prognosis. Our results suggest HSF1 as a promising molecular target for the development of anti-HCC therapeutics.

Abbreviations: BAG3, bcl-2-associated athanogene domain 3; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; HSF1, heat shock factor 1; HSF1 KD, HSF1 knockdown; HSP, heat shock protein; IKKγ, IκB kinase gamma; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mRNA, messenger RNA; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; SCID, severe combined immune-deficient mice; shRNA, small hairpin RNA; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.

Materials and methods

Cell cultures and reagents

Human HCC cell lines HepG2, PLC/PRF/5, HLE and HLF were obtained from the American Type Culture Collection. Huh7 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). KIM-1 and KYN2 were kindly provided by Dr Hirohisa Yano (Department of Pathology, Kurume University, Kurume, Japan). Li7 was kindly provided by Dr Yae Kanai (Division of Molecular Pathology, National Cancer Center Research Institute,

Tokyo, Japan). HepG2, PLC/PRF/5, Huh7, HLE and HLF cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. KIM-1 and KYN2 was maintained in RPMI medium containing 10% fetal bovine serum.

Antibodies and chemicals

The antibodies used included: anti-HSF1, ERK1/2, phosphor-ERK1/2, MAPK kinase (MEK), phospho-MEK, phosphor- efficiently activated epidermal growth factor receptor (EGFR), cyclin D1, cdc2, CDK4, phospho-IκBα, IκB kinase gamma (IKKγ), IKKβ, caspase-3 and Bcl-X_L (Cell Signaling Biotechnology, Danvers, MA); anti-HSP90, HSP72, β-actin and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-EGFR (Millipore, Billerica, MA); anti-HSP70/HSP72 (Enzo Life science, NY); and anti-BAG3 (Abcam, Cambridge, UK).

Biochemical and immunohistochemical analyses

Protein lysates were prepared from tissues and cultured cells, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto Immobilon membranes (Millipore) and analyzed by immunoblotting. Total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), then cDNA was synthesized using SuperScript II (Invitrogen), and expression of specific messenger RNAs (mRNAs) was quantified using real-time PCR and normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA expression. Details of real-time PCR conditions and primer sequences are available in Supplementary Materials and methods, available at *Carcinogenesis* Online. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using immunoperoxidase methods, as described previously (15). For array analysis, we used the Human WG-6 BeadChip-kit (Illumina, San Diego, CA) in accordance with the instructions from the manufacturer (details are given in Supplementary Materials and methods, available at *Carcinogenesis* Online).

Establishment of HSF1-knockdown cells

A HSF1 small hairpin RNA (shRNA) plasmid and negative control plasmid were purchased from SABiosciences (QIAGEN, Valencia, CA). The shRNA sequences targeting HSF1 were from position 5′-CAGGTTGTTCATAGTCAGAAT-3′ as in the nucleotide sequence of HSF1. As a negative control, a shRNA was designed with the sequence 5′-GGAATCTCATTCGATGCATAC-3′. Transfection was achieved using Oligofectamine reagent (Invitrogen) according to the instructions from the manufacturer. To establish stable knockdown cell lines, shRNA plasmids were transfected into KYN2 cells and cultured in the presence of puromycin (Sigma–Aldrich, St Louis, MO).

Cell proliferation and bromodeoxyuridine assay

Cell proliferation in response to HSF1 silencing was determined by trypan blue exclusion assay. DNA synthesis was determined by bromodeoxyuridine assay according to the instructions from the manufacturer (Roche Diagnostics, Basel, Switzerland). The result was expressed as a percentage of the maximum absorbance at 450 nm, based on three independent experiments. Cells were counted using a Coulter Counter (Beckman Coulter, Pasadena, CA).

Apoptosis assay

Assessment of apoptosis was performed by measuring the intensity of the sub-G_1 peak. For the sub-G_1 peak, HSF1 control KYN2 cells or HSF1-knockdown (HSF1 KD) KYN2 cells were tumor necrosis factor alpha (TNF-α) treatment for 24 h. Cells were treated with propidium iodide and then the sub-G_1 peak was analyzed with a fluorescence-activated cell sorting (FACS) flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed in accordance with the manufacturer's instructions (ApopTag kit; Intergen, Burlington, MA).

Animals

HSF1-deficient ($HSF^{-/-}$) mice have been described previously (29). C57BL/6 wild-type (WT) mice were purchased from CLEA Japan (Tokyo, Japan) for use in the experiments, with primary hepatocytes isolated using a collagenase perfusion method as described in a previous report (26). For orthotopic implantation, C.B-17/Icr-scid/scidJcl [severe combined immune-deficient mice (SCID)] mice were obtained from CLEA Japan. All mice were maintained in filter-topped cages on autoclaved food and water at the University of Hokkaido and the Institute for Adult Diseases, Asahi Life Foundation, according to National Institutes of Health (NIH) guidelines. All experimental protocols were approved by the ethics committee for animal experimentation at Hokkaido University and Asahi Life Foundation. Orthotopic implantation of KYN2 cells and KYN2 transfectants were performed as described previously (30). Briefly, mice were inoculated orthotopically with 5×10^6 HSF1 control ($n = 12$) and HSF1 KD ($n = 12$) cells in 100 μl of phosphate-buffered saline, injected into the liver. Mice were killed 6 weeks after inoculation and autopsies were performed immediately. In the lipopolysaccharide (LPS)/D-galactosamine (GalN)-induced liver injury model, mice were injected intraperitoneally with LPS (20 lg/kg; Sigma) and GalN (1000 mg/kg; Wako, Osaka, Japan) (24).

Patients and tissue samples

For immunohistochemical analysis, a total of 226 adult patients with HCC who underwent curative resection between 1997 and 2006 at Hokkaido University Hospital were enrolled in this study. A preoperative clinical diagnosis of HCC was required to meet the diagnostic criteria of the American Association for the Study of Liver Diseases. Briefly, inclusion criteria were as follows: (i) distinctive pathological diagnosis, (ii) no preoperative anticancer treatment or distant metastases, (iii) curative liver resection (exclusion of extrahepatic tumor spread/metastasis) and (iv) complete clinicopathological and follow-up data. The study protocols were approved by the institutional review board and performed in compliance with the Helsinki of Declaration. Written informed consent was obtained from as many of the patients who were alive as possible (deceased cases were approved for use without written informed consent). Histological diagnosis was made according to World Health Organization criteria. The main clinicopathological features are presented in Table I. During follow-up, clinical evaluations and biochemical tests were performed every 1–3 months. Patients underwent triphasic computed tomography of the liver every 2–3 months.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Significant differences were detected using non-parametric testing. Correlations between protein expression and clinicopathological features of the specimens were assessed, and the resulting data were analyzed using the χ^2 test and Fisher's exact test. Cumulative survival rate was calculated from the first date of treatment using the Kaplan–Meier life-table method. Differences were evaluated by log-rank testing. Independent factors for survival were assessed with the Cox proportional hazard regression model. Differences between the two groups were analyzed using the log-rank test. Statistical analyses were performed using Stat View software (version 5.0; SAS Institute, Cary, NC). Values of $P < 0.05$ were considered significant.

Results

Effect of HSF1 on tumor growth

We first investigated expression of HSF1 in cultured HCC cell lines. HSF1 expression was detected in all eight HCC cell lines analyzed. KYN2 cells showed significantly higher expression of HSF1 than other cell lines (Figure 1A). To further elucidate the functional role of HSF1 in HCC, we established HSF1 KD KYN2 cells by expressing the shRNA against HSF1 or control shRNA. To evaluate the effects of HSF1 on cell growth, we measured cell numbers at several time points and found that the growth of HSF1 KD cells was significantly inhibited compared with control cells (HSF1 control) (Figure 1B). Cell cycle regulators including PCNA, cyclin D1, cdc2 and CDK4 were suppressed in HSF1 KD cells compared with HSF1 control cells (Figure 1C). These results indicate that HSF1 enhances HCC cell growth. Concordantly, HSF1 KD reduced DNA synthesis as measured by bromodeoxyuridine incorporation (Figure 1D).

To evaluate the effects of HSF1 on HCC *in vivo*, orthotopic xenografts were established by HSF1 control and HSF1 KD KYN2 cells in nude mice. Maximum primary tumor diameters and tumor volumes were significantly decreased in HSF1 KD xenografts compared with HSF1 control ones (Figure 1E), suggesting that HSF1 accelerated HCC tumor growth *in vivo*. We confirmed that the tumor of HSF1 KD cells showed significantly lower expression of HSF1 and PCNA than the tumor of HSF1 control cells (Figure 1E).

We performed gain-of-function experiments for HSF1 *in vitro*. No apparent changes in cell growth were seen with overexpression of HSF1 in HCC cell lines with low HSF1 expression (Supplementary Figure 1, available at *Carcinogenesis* Online), whereas cell growth was reduced in HSF1 KD experiments, as above. Based on these

Table I. HSF1, BAG3 expression and clinicopathological variables in HCC

Parameter	Total	HSF1		*P*	BAG3		*P*
		High	Low		High	Low	
		n = 115	*n* = 111		*n* = 112	*n* = 114	
		≥30	<30		≥25	<25	
Age (years)							
≥60	126	66	60	0.69	59	67	0.42
<60	100	49	51		53	47	
Sex							
Male	185	95	90	0.86	94	91	0.49
Female	41	20	21		18	23	
Etiology							
HBsAg(+)/HCV(−)	85	45	40	0.70	39	46	0.67
HBsAg(−)/HCV(+)	84	43	41		44	40	
HBsAg(+)/HCV(+)	6	4	2		2	4	
HBsAg(−)/HCV(−)	51	23	28		27	24	
Cirrhosis							
Presence	121	64	57	0.59	62	59	0.59
Absence	105	51	54		50	55	
Tumor size (cm)							
<5	149	67	82	0.017*	66	83	0.035*
≥5	77	48	29		46	31	
No. of tumor nodules							
Solitary	168	78	90	0.032*	79	89	0.22
Multiple (≥2)	58	37	21		33	25	
TNM stage							
I and II	139	62	77	0.017*	63	76	0.11
III and IV	87	53	34		49	38	
BCLC stage							
A	81	27	54	<0.001*	32	49	0.065
B	108	64	44		58	50	
C	37	24	13		22	15	
Differentiation							
Well	36	11	25	0.010*	10	26	0.014*
Moderate	143	74	69		75	68	
Poor	47	30	17		27	20	
Capsular formation							
Presence	184	95	89	0.73	91	93	1.0
Absence	42	20	22		21	21	
Vascular invasion							
Present	37	24	13	0.073	22	15	0.21
Absent	189	91	98		90	99	
Serum AFP level							
<20	117	53	64	0.086	52	65	0.14
≥20	109	62	47		60	49	

AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HCV, hepatitis C virus; TNM, tumor node metastasis.
*Significant *P* value.

findings, we concluded that HSF1 expression is a necessary condition for cell growth, but it is not a sufficient condition. We, therefore, did not further investigate gain of function of HSF1.

Impaired EGF-mediated MEK/ERK activation in HSF1 KD cells and HSF1$^{-/-}$ hepatocytes

Activation of the MEK/ERK pathway regulates many important cellular processes in carcinogenesis. To further elucidate the function of HSF1 on tumor growth, we investigated the cascade of MAPK. In WT hepatocytes and HSF1 control cells, EGF, a potent activator of MAPK, efficiently activated EGFR, MEK1/2 and ERK1/2 (Figure 2A). In contrast, activation of EGFR, MEK1/2 or ERK1/2 was significantly decreased in HSF-knockout mice (HSF$^{-/-}$) hepatocytes and HSF1 KD cells (Figure 2A and B). Regarding protein levels of EGFR, MEK1/2 and ERK1/2, EGFR protein levels were significantly decreased in HSF1$^{-/-}$ hepatocytes and HSF1 KD compared with controls, whereas other proteins were unchanged (Figure 2A and B). This result was consistent with the previous report (31). Immunohistochemical staining revealed that HSF1 control tumor showed strong phosphorylated ERK1/2 levels, whereas almost no ERK1/2 activation was observed in HSF1 KD tumors (Figure 2C).

Role of HSF1 in TNF-α-induced apoptosis

Since tumor growth inhibition is caused mainly by increased cell death and decreased cellular proliferation, we compared numbers of apoptotic cell deaths in HSF1 control and HSF KD xenografts using the TUNEL assay. Significantly more apoptotic tumor cells were found in HSF1 KD tumors than in HSF1 control tumors (Figure 3A). Next, we examined whether HSF1 was involved in apoptosis *in vitro*. FACS analysis showed very few apoptotic cells in HSF KD or HSF control in the absence of any stimuli. In contrast, treatment with TNF-α, a potent inducer of apoptosis, caused more extensive apoptotic cell death in HSF1 KD cells (23.9%) than in HSF control cells (8.7%) (Figure 3B). Furthermore, we also confirmed increased TNF-α-induced apoptosis in HSF KD cells as determined by TUNEL assay and caspase-3 activation (Figure 3C and D). To examine whether HSF1 is required for TNF-α-induced liver apoptosis *in vivo*, we used an LPS/GalN liver injury model that depends on TNF-α-mediated apoptosis (32). At 7 h LPS/GalN