Table 4 Univariate analysis of pretreatment factors associated with interferon-induced neutropenia

	Case-G2 + R[a] ($n = 100$)	Control-G + R[b] ($n = 656$)	P value[c]
Gender, male/female	45/55	378/278	0.018
Age, years	58.1 (9.3)	56.9 (10.4)	0.262
Neutrophil count, /mm^3	1,614 (735)	2,742 (979)	<0.001
Hemoglobin, g/dL	13.5 (1.5)	14.2 (1.5)	<0.001
Platelet count, $\times 10^9$/L	136 (46)	163 (57)	<0.001
ALT, IU/L	79.1 (69.7)	83.5 (74.3)	0.574
HCV RNA, log IU/ml	6.0 (0.9)	6.1 (0.8)	0.164
Liver fibrosis, F0-2/F3-4/ND	46/16/38	397/157/102	0.674
rs2305482, AA + AC/CC/ND	74/24/2	591/59/6	<0.001
rs4794822, TT + TC/CC/ND	56/42/2	484/130/42	<0.001

Data are expressed as number for categorical data or the mean (standard deviation) for non-categorical data

ALT alanine transaminase, *ND* not determined

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Categorical variables were compared between groups by the Chi square test and non-categorical variables by the Student's t test

Table 5 Logistic regression analysis of pretreatment factors associated with interferon-induced neutropenia

	OR (95 % CI)	P value
Gender, female	1.229 (0.734–2.059)	0.4331
Neutrophil count, /mm^3	0.998 (0.997–0.998)	<0.0001
Platelet count, $\times 10^9$/L	1.005 (0.953–1.059)	0.8604
rs2305482, CC	2.497 (1.281–4.864)	0.0072

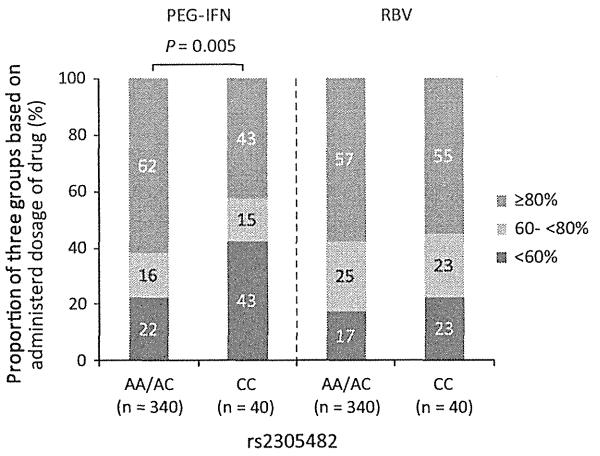

Fig. 3 Administered doses of PEG-IFN and RBV according to rs2305482 genotypes. The patients were stratified into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients receiving <60 % of the PEG-IFN doses was significantly higher in patients with rs2305482 CC than in those with AA/AC ($P = 0.005$, by the Chi square test). *PEG-IFN* pegylated interferon, *RBV* ribavirin

reasons for the dose reduction of PEG-IFN in PEG-IFN/RBV therapy. While, there were no associations between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

PSMD3 encodes the proteasome 26S subunit, non-ATPase 3, a member of the 26S proteasome family, and is involved in the control of cell cycle transition via the ubiquitin–proteasome pathway (Bailly and Reed 1999). *CSF3* encodes G-CSF, which controls the production, differentiation, and function of granulocytes (Nagata et al. 1986). Recombinant G-CSF is widely used to treat patients with severe neutropenia during chemotherapy. Therefore, we hypothesize that *PSMD3-CSF3* variants may influence neutrophil counts through affecting the process of endogenous G-CSF synthesis during IFN-based therapy or other bone marrow suppressive therapies. However, eQTL analysis by Okada et al. (2010) showed that rs4794822 was significantly associated with the expression level of *PSMD3*, rather than that of *CSF3* in the JPT and CHB populations. Our eQTL analysis showed that the risk allele for neutropenia at rs2305482 correlated with higher expression levels of *PSMD3* in LWK and MEX populations (Supplementary Fig. 5a), whereas with lower expression levels of *CSF3* in MEX and especially in CHB populations (Supplementary Fig. 5b, c). However, these results were not replicated in the other probe of *CSF3*. Additionally, we analyzed serum G-CSF levels in CHC patients receiving IFN-based therapy. Although serum G-CSF levels were thought to be increased in response to neutropenia regardless of rs2305482 and rs4794822 genotypes, there was no evidence that they were lower in patients with a risk allele of these SNPs at baseline and during the neutropenic period (Supplementary Fig. 6). Moreover, neutrophil counts did not correlate with serum

G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a). Further functional analyses of these genes and polymorphisms are required to elucidate the reason for the association between *PSMD3-CSF3* and IFN-induced neutropenia as well as neutrophil counts in healthy individuals.

In previous reports, *PLBC4*, *DARC*, *CXCL2*, and *CDK5* loci have also been associated with neutrophil or WBC counts in healthy individuals or patients who were not under chemotherapy (Crosslin et al. 2012; Kamatani et al. 2010; Okada et al. 2010; Reiner et al. 2011). However, there were no associations with these loci discernible in our GWAS.

The important limitation of this study is that the association between rs2305482 and IFN-induced neutropenia was not statistically significant in a genome-wide level. Thompson et al. (2012) also identified no genetic determinants of IFN-induced neutropenia during PEG-IFN/RBV therapy at the level of genome-wide significance by their GWAS. Unlike our study design, they analyzed the association between the reduction of neutrophil counts at week 4 and any SNPs. Indeed, we analyzed the association between the reduction of neutrophil counts at week 2 or 4 and rs2305482 or rs4794822, but there was no significant association. Therefore, further independent replication analyses which are designed in the similar way as our study are desirable.

IFN-free therapies are expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. However, combination therapies of DAA and IFN will continue to be used for some time. Our findings contribute to our understanding of the genetic factors influencing IFN-induced neutropenia. Furthermore, these genetic variants may be associated with neutropenia during chemotherapies for various malignant diseases as well as IFN-based therapy for CHC. Therefore, genetic testing of these variants might be useful for establishing personalized doses of such therapies to minimize drug-induced adverse events. Additionally, our results might contribute to the elucidation of the mechanism of drug-induced neutropenia.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Pancreatic congestion in liver cirrhosis correlates with impaired insulin secretion

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Abstract
Background Although impaired glucose tolerance is common in cirrhosis, this condition's pathogenesis remains undefined. This study aimed to clarify pathogenesis related to the pancreas in cirrhotic patients, and to evaluate associations between insulin secretion and pancreatic congestion due to portal hypertension.
Methods Pancreatic perfusion parameters were analyzed by dynamic contrast-enhanced ultrasound (CE-US) in 41 patients (20 cirrhotic, 21 non-cirrhotic; age, 67.9 ± 13.3; female, 19), and prospectively compared to delta C-peptide immunoreactivity (ΔCPR). In a separate study, a retrospective chart review with human autopsy specimens was conducted, and vessels and islets of the pancreas were analyzed in 43 patients (20 cirrhotic, 23 controls; age, 71.5 ± 11.6; female, 15).
Results In the CE-US study, the clinical characteristics indicative of portal hypertension (e.g., ascites and varices) had significantly higher incidences in the cirrhotic group than in the control group. Pancreatic drainage times were greater in the cirrhotic group ($p < 0.0001$), and had a significant negative correlation with ΔCPR ($R = 0.42$, $p = 0.0069$). In the histopathological study, the islets were enlarged in the cirrhotic group ($p < 0.0001$). However, the percentage of insulin-positive area per islet was decreased in the cirrhotic group ($p < 0.0001$), and had a significant negative correlation with the wall thickness of the pancreatic vein ($R = 0.63$, $p < 0.0001$).
Conclusions Pancreatic congestion was present in cirrhotic patients. Moreover, pancreatic congestion and insulin secretion were significantly correlated. This pathogenesis could be a key factor underlying the development of hepatogenous diabetes in cirrhotic patients.

Keywords Portal hypertension · Pancreas · Congestion · Liver cirrhosis · Contrast-enhanced ultrasound

Abbreviations

LC	Liver cirrhosis
PDX-1	Pancreatic duodenal homeobox-1
CE-US	Contrast-enhanced ultrasound
B-RTO	Balloon-occluded retrograde transvenous obliteration
BMI	Body mass index
ΔCPR	Delta C-peptide immunoreactivity

Introduction

Patients with liver cirrhosis (LC) often present with a history of impaired glucose tolerance, which is known as hepatogenous diabetes. Hepatogenous diabetes can result

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in both critical blood glucose abnormalities (e.g., hypoglycemia and hyperglycemia) and decreased immunocompetence. However, because LC patients typically experience liver metabolic disorders and malnutrition, the treatment of hepatogenous diabetes is often difficult. Moreover, although hyperinsulinemia and insulin resistance are its characteristic clinical conditions, the pathogenesis of hepatogenous diabetes remains undefined. Nonetheless, several previous studies have suggested that intra- and extra-hepatic portosystemic shunts might contribute to a decrease in hepatic insulin clearance and thereby lead to hyperinsulinemia [1–5]. In addition, the findings of several animal studies and a postmortem human study suggest that hyposecretion may occur in LC patients, even as glucose tolerance is impaired. Although hyperinsulinemia developed in rats with partial portal vein ligation or carbon tetrachloride-induced cirrhosis, insulin secretion of the pancreatic islets decreased [6, 7]. Meanwhile, the expression of insulin was reduced in postmortem pancreatic tissue obtained from LC patients, while the expression of a pancreatic transcription factor and pancreatic duodenal homeobox-1 (PDX-1) was increased [8]. Thus, the identification of a mechanism providing a link between hepatic clearance and a decrease in insulin secretion could yield a new understanding of the pathogenesis of hepatogenous diabetes.

Some studies have reported that LC patients develop congestive splenomegaly due to portal hypertension [9–12]. Moreover, because pancreatic drainage blood flow drains to a portal system, the pancreas may also exhibit congestion due to portal hypertension [13, 14]. Congestive changes have also been reported in the pancreatic tissue of patients with chronic heart failure [15]. Thus, we hypothesized that pancreatic congestion due to portal hypertension might influence the function of the pancreas and thereby result in hepatogenous diabetes.

On the basis of contrast-enhanced ultrasound (CE-US) results, the present study aimed to prospectively evaluate the association between insulin secretion and pancreatic congestion due to portal hypertension in LC patients (the CE-US study component). Additionally, we aimed to evaluate pancreatic status in LC patients, to determine whether congestive changes were evident (the pathological study component).

Methods

Study design

The CE-US study was a prospective cohort study conducted at Ehime University Hospital (Ehime, Japan). The pathological study was a retrospective study conducted at two institutions in Ehime, Japan: Ehime University Hospital and Saiseikai Imabari Hospital. This study protocol was approved by the relevant institutional ethics committee (Approval Number: 1302010), and written informed consent was obtained from the patients. This study was registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (Registration Number: 000010790).

Patients

The CE-US study

Consecutive patients who were hospitalized in Ehime University hospital and who were aged ≥20 years were enrolled prospectively between April 2012 and July 2013. The patients were divided into an LC group and a control group. In the LC group, all patients had a histologically proven diagnosis of LC based on liver biopsy. The control group had no past medical history of pancreatic disease or diabetes. For both groups, patients were excluded if they had laboratory increases in pancreatic enzyme (amylase >200 IU/l and/or lipase >49 IU/l), had morphologic changes in the pancreas (e.g., pancreatic tumors, cysts, enlargement, calcification, or dilatation/narrowing of the main pancreatic duct) reported on abdominal imaging (helical computed tomography, magnetic resonance cholangiopancreatography, and ultrasonography), had past treatment history related to portal hypertension [splenectomy, partial splenic embolization, or balloon-occluded retrograde transvenous obliteration (B-RTO)], had diabetes (fasting plasma glucose of <110 mg/dl and/or glycated hemoglobin of <6.0 %), consumed >20 g of alcohol per day, were administered beta-blockers, or had a clinical condition that could have caused portal hypertension, including chronic heart failure and portal vein tumor thrombosis. Patients were also excluded if a pancreatic ultrasound image had not been obtained. CE-US and the glucagon challenge test were performed in all eligible patients, and the results were compared between the two groups.

The pathological study

A retrospective chart review was conducted and included 43 consecutive patients who underwent autopsy at Ehime University Hospital or Saiseikai Imabari Hospital between April 2000 and October 2012. The patients were also divided into an LC group and a control group. Patients were excluded if they had anatomical changes in the pancreas; had a past medical history of diabetes, splenectomy, partial splenic embolization, or B-RTO; consumed >20 g of alcohol per day; were administered beta-blockers; or had a clinical condition that could have caused portal hypertension, including chronic heart failure and portal vein

tumor thrombosis. The wall thickness of the pancreatic vein, the diameters of the islet cells, the percentages of insulin- or glucagon-positive area per islet, the number of insulin-positive cells, and the percentage of insulin-positive cells per islet (from the pancreas specimens that were obtained) were compared between the two groups.

Data collection

Relevant clinical data (e.g., age, sex, weight, height, alcohol intake, and smoking history) and past medical history (e.g., hypertension, dyslipidemia, ascites, or varices) were collected from all patients on admission. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

In the CE-US study, venous blood samples were obtained after patients had fasted overnight (>12 h), and splenic volume (cm^3) and the diameter of the portal vein (mm) were measured by dynamic computed tomography imaging. Blood cell count, biochemical data, coagulation profiles, and serological data were obtained before performing CE-US. After performing CE-US, the glucagon challenge test was performed.

In the pathological study, biochemical data collected on admission were retrieved from the chart. Splenic volume (g) was measured at the time of the pathologic anatomical study.

Glucagon challenge test

The glucagon challenge test, which evaluates the insulin response to elevation of blood glucose induced by the administration of glucagon, was performed to estimate the β-cell function of the islet. All glucagon challenge tests were performed by one blinded investigator. One milligram of glucagon (Glucagon G Novo; Novo Nordisc, Tokyo, Japan) in 8 ml of 10 % saline was injected as a bolus within 30 s. Fifteen seconds after the initiation of the intravenous administration was considered to be the start at 0 min for subsequent blood collection. The blood samples were collected at four points: baseline (before the glucagon injection) and at 3, 6, and 10 min after the glucagon injection. The serum levels of C-peptide were measured at each point. The differences between the maximum and baseline C-peptide values were subsequently determined, and were defined as the delta C-peptide immunoreactivity (ΔCPR).

CE-US

CE-US was performed during hospitalization by one blinded ultrasound specialist who had 15 years of experience (he had performed >50 CE-US studies of the pancreas prior to this study) after the patients had fasted overnight >12 h. CE-US was performed with an APLIO scanner (APLIO 500 TUS-A500; Toshiba Medical Systems, Tokyo, Japan) and a 3.5-MHz convex-array. Harmonic Imaging Quantification software (Toshiba Medical Systems, Tokyo, Japan) was used to determine the region of interest. Vascular imaging was performed using the vascular recognition imaging mode, in which gray-scale-coded fundamental B-mode imaging provided anatomic information. Perflubutane microbubble agent (Sonazoid; Daiichi-Sankyo, Tokyo, Japan) was used as the contrast media.

The investigator visualized the pancreas body and splenic vein by epigastric wide scanning and defined a region of interest with an area of approximately 5 mm × 5 mm, subsequently focusing on the region of the pancreatic body nearest to the body surface. An inflow of contrast media (0.015 ml/kg perflubutane injected as a bolus) was then evaluated. After the results were converted

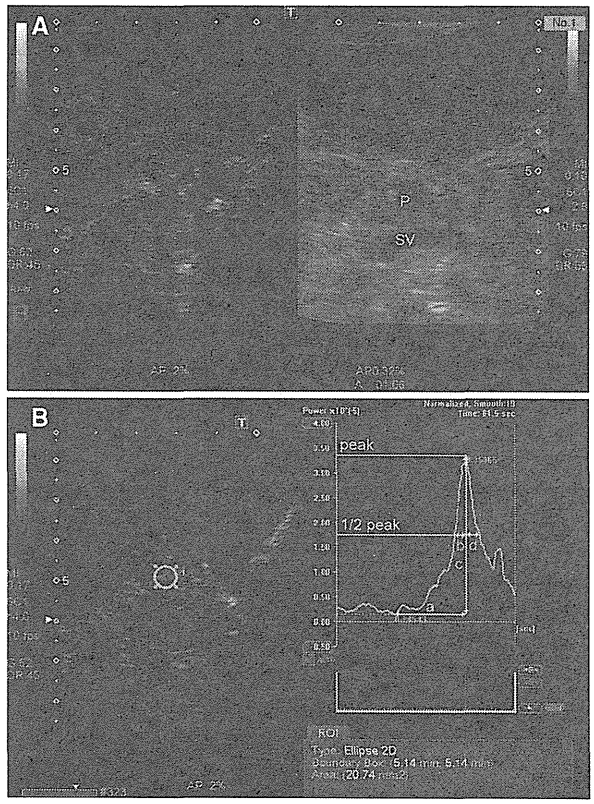

Fig. 1 Pancreatic contrast-enhanced ultrasound (CE-US) images and time–intensity curves of pancreatic blood flow. **a** The *right image* is a B-mode image; the *left image* represents the vascular recognition imaging mode. The investigator visualized the pancreas body (P) and splenic vein (SV) using an epigastric wide scan. **b** The *left image* presents the vascular recognition imaging mode; the *right image* presents the time–intensity curve of pancreatic blood flow. The *circle* indicates the region of interest in the pancreas body at a position nearest to the body surface. The time to peak (*a*), time from 1/2 peak to peak of the wash-in phase (*b*), peak intensity (*c*), and time from peak to 1/2 peak of the wash-out phase (*d*) were analyzed

into a time–intensity curve, the time to peak, time from 1/2 peak to peak in the wash-in phase, peak intensity, and time from peak to 1/2 peak in the washout phase were analyzed (Fig. 1a, b).

Immunohistochemistry

Pancreatic tissues with a pathological anatomy were fixed in formalin. Sections (3-μm thickness) were cut from each block, and adjacent sections were stained with standard hematoxylin-eosin, Elastica–Masson, Picrosirius Red, and immunohistochemical staining techniques. Paraffin-embedded samples were dewaxed and rehydrated, and endogenous peroxidase activity was inactivated by incubation with methanol containing 1 % hydrogen peroxidase for 20 min. The sections were then incubated in 1 % blocking goat serum for 30 min to reduce nonspecific reactions. For immunohistochemistry, the sections were incubated with the relevant primary antibody (Anti-insulin antibody; Nichirei 418091, Tokyo, Japan, and Anti-glucagon antibody; Nichirei 422271, Tokyo, Japan) at 4 °C overnight. The tissue sections were treated with peroxidase-labeled secondary antibody (Histofine Simple stain Max PO; Nichirei, Tokyo, Japan) for 1 h at room temperature, and incubated with Simple Stain DAB Solution (Nichirei).

Pathological analyses

Slides were imaged in bright field using the NanoZoomer Digital Pathology System (Hamamatsu Photonics, K.K., Japan). Collagen type 1 and type 3 in pancreatic venous walls were stained with Picrosirius Red and observed by polarization microscopy. Immunohistochemistry expression analyses were performed using ImageJ software [16, 17]. All pathological analyses were performed by two blinded pathologists who each had 9 years of experience, and were conducted if at least five vessels and five islets were present in each pancreatic specimen. The pathologists measured the wall thickness of the pancreatic vein, the diameter of the islet cells, the percentage of insulin- or glucagon-positive area per islet, the number of insulin-positive cells, and the percentage of insulin-positive cells per islet. The resulting values are expressed as the median measurements.

Outcome measures

The CE-US study

The primary outcome measure was a correlation of the time–intensity curve derived from the CE-US results (time from peak to 1/2 peak in the washout phase, which reflects drainage time from the pancreas) and ΔCPR. The secondary outcome measures included time to peak, time from 1/2 peak to peak of the wash-in phase, peak intensity, time from peak to 1/2 peak of the washout phase, the diameter of the portal vein, and ΔCPR.

The pathological study

The primary outcome measure was the correlation between the wall thickness of the pancreatic vein and the percentage of the insulin-positive area per islet. The secondary outcome measures included the wall thickness of the pancreatic vein, the diameter of the islet cell, the percentage of insulin- and glucagon-positive area per islet, the number of insulin-positive cells, and the percentage of insulin-positive cells per islet.

Statistical Analyses

All results are expressed as the mean ± 1 standard deviation. Data were analyzed using Student's *t* test for unpaired data and Fisher's exact test, as appropriate. The following relationships were analyzed using the Pearson product–moment correlation coefficient: the relationship between time from peak to 1/2 peak and ΔCPR, and the relationship between the wall thickness of the pancreatic vein and the insulin- or glucagon-positive areas. All analyses were performed using JMP software (version 8; SAS Institute Japan, Tokyo, Japan).

Results

Patients

The CE-US study

During the study period, a total of 54 patients were enrolled in the study (LC group, 29 patients; control group, 25 patients). Of these, 13 patients were excluded from the study: four patients (three in the LC group and one in the control group) were excluded because of alcohol consumption exceeding 20 g; seven patients (four in the LC group and three in the control group) were excluded because of diabetes; and two patients (in the LC group only) were excluded because a pancreatic ultrasound image could not be obtained as a result of severe intestinal gas. Finally, 41 eligible patients (LC group, 20 patients; control group, 21 patients) were included and analyzed (S1). The characteristics of the two groups are shown in Table 1. In the LC group, 12 and eight patients were classified into grade A and grade B, respectively, according to the Child-Pugh classification. The etiology of the LC group was as

Table 1 Demographics and laboratory data of the patients enrolled in the contrast-enhanced ultrasound study ($n = 41$)

Characteristic	Liver cirrhosis ($n = 20$)	Control ($n = 21$)	p value
Age (years)	70.5 ± 3.0	65.5 ± 2.9	0.23
Sex (female)	10	9	0.65
BMI (kg/m^2)	22.4 ± 0.7	22.7 ± 0.7	0.74
Smoking	7	8	0.84
Hypertension	9	10	0.87
Dyslipidemia	1	3	0.32
Ascites	9	0	0.0005
Esophageal varices	10	0	0.0003
Splenic volume (cm^3)	304.0 ± 34.0	127.5 ± 33.2	0.0006
ALT (IU/l)	51.3 ± 9.0	40.0 ± 8.8	0.38
Total bilirubin (mg/dl)	1.1 ± 0.1	0.7 ± 0.1	0.0066
Albumin (g/dl)	3.3 ± 0.1	3.8 ± 0.1	0.028
Prothrombin time (%)	76.3 ± 4.6	90.0 ± 4.7	0.044
Platelet count (10^4/μl)	12.4 ± 1.6	19.5 ± 1.5	0.0024
Creatinine (mg/dl)	0.9 ± 0.1	0.8 ± 0.1	0.13
HbA1c (%)	5.2 ± 0.1	5.5 ± 0.1	0.17
Plasma glucose (mg/dl)	98.5 ± 3.5	95.3 ± 3.4	0.53
Insulin (μU/ml)	13.8 ± 1.4	10.2 ± 1.3	0.062
CPR/IRI molar ratio	26.1 ± 3.5	22.5 ± 3.4	0.46
HOMA-IR	3.5 ± 0.5	2.5 ± 0.4	0.11
Child-Pugh grade: A/B/C	12/8/0	NA	NA
Etiology/medical condition (number of patients)	HCV (9)	Biopsy proven non-LC (6);	NA
	HBV (3)	HCV (5), NASH (1)	
	HBV + HCV (1)	Gastrointestinal disease (8)	
	PBC (3)	Choledocholithiasis/intrahepatic	
	AIH (1)	Cholelithiasis[a] (4)	
	NASH (2)	Others (3)	
	Amyloid disease (1)		

Values are given as number of patients or mean ± standard deviation

CPR/IRI molar ratio = basal C-peptide immunoreactivity (mmol/l)/basal immunoreactive insulin (pmol/l)

HOMA-IR = basal insulin (μU/ml) × basal plasma glucose (mg/dl)/405

BMI body mass index, *ALT* alanine aminotransferase, *HbA1c* glycated hemoglobin, *CPR* C-peptide immunoreactivity, *IRI* immunoreactive insulin, *HOMA-IR* homeostasis model assessment of insulin resistance, *HCV* hepatitis C virus, *HBV* hepatitis B virus, *PBC* primary biliary cirrhosis, *AIH* autoimmune hepatitis, *NASH* non-alcoholic steatohepatitis, *LC* liver cirrhosis, *NA* not applicable

[a] No history of cholangitis

follows: hepatitis C (nine patients), hepatitis B (three patients), hepatitis B+C (one patient), primary biliary cirrhosis (three patients), autoimmune hepatitis (one patient), nonalcoholic steatohepatitis (two patients), and liver amyloid disease (one patient). The medical condition of the control group was as follows: biopsy-proven non-cirrhotic liver disease (hepatitis C; five patients), non-alcoholic steatohepatitis (one patient), gastrointestinal disease (eight patients), choledocholithiasis or intrahepatic cholelithiasis (no history of cholangitis; four patients), and others (three patients). The following baseline characteristics were similar between the LC and control groups: age, sex, BMI, smoking history, and past medical history of hypertension or dyslipidemia. In the LC group, the incidences of characteristics suggestive of portal hypertension (e.g., ascites, varices, and the splenic volume) were significantly higher than those in the control group ($p = 0.0005$, $p = 0.0003$, and $p = 0.0006$, respectively). In regards to liver function, total bilirubin was significantly higher in the LC group than in the control group ($p = 0.0066$), whereas albumin and prothrombin time were significantly lower in the LC group than in the control group ($p = 0.028$ and $p = 0.044$, respectively). In addition, the basal C-peptide to insulin molar ratio, which suggests the presence of portosystemic shunts, did not differ between the two groups ($p = 0.46$). Further, the

Table 2 Demographics and laboratory data of patients enrolled in the pathological study ($n = 43$)

Characteristic	Liver cirrhosis ($n = 20$)	Control ($n = 23$)	p value
Age (years)	68.5 ± 2.5	74.0 ± 2.4	0.12
Sex (female)	7	8	0.99
BMI (kg/m^2)	23.6 ± 1.1	20.6 ± 1.0	0.053
ALT (IU/l)	132.2 ± 35.1	22.3 ± 32.7	0.027
Total bilirubin (mg/dl)	8.8 ± 2.0	0.7 ± 1.9	0.0051
Albumin (g/dl)	3.0 ± 0.1	3.2 ± 0.1	0.48
Platelet count (10^4/μl)	11.3 ± 2.2	22.8 ± 2.0	0.0004
Plasma glucose (mg/dl)	114.5 ± 6.0	117.2 ± 5.6	0.74
Splenic volume (g)	272.8 ± 32.1	86.6 ± 29.9	0.0001
Etiology/medical condition (number of patients)	HCV (15)	Respiratory disorder (10)	NA
	HBV (3)	Acute coronary syndrome (6)	
	PBC (2)	Gastrointestinal disease (4)	
		Infectious disease (2)	
		Chronic renal failure (1)	

Values are given as number of patients or mean ± standard deviation

BMI body mass index, *ALT* alanine aminotransferase, *HCV* hepatitis C virus, *HBV* hepatitis B virus, *PBC* primary biliary cirrhosis

homeostasis model assessment of insulin resistance, which is a method of quantifying insulin resistance, did not differ between the two groups ($p = 0.11$).

The pathological study

A total of 43 autopsied patients (LC group, 20 patients; control group, 23 patients) were eligible for this study. The etiology of the LC group was as follows: hepatitis C (15 patients), hepatitis B (three patients), and primary biliary cirrhosis (two patients). The medical condition of the control group was as follows: respiratory disorder (ten patients), acute coronary syndrome (six patients), gastrointestinal disease (four patients), infectious disease (two patients), and chronic renal failure (one patient). No differences were observed between the two groups in terms of clinical characteristics (e.g., age, sex, and BMI). Total bilirubin and splenic volume were significantly higher in the LC group ($p = 0.0001$, Table 2).

Diameter of the portal vein

As the pancreatic perfusion parameter, the diameter of the portal vein was significantly greater in the LC group than in the control group (14.6 ± 0.5 vs. 9.9 ± 0.5 mm, $p < 0.0001$).

Enhancement pattern of the pancreas on CE-US

The inflow and outflow times of the contrast media in the pancreas were significantly slower in the LC group than those in the control group: time to peak (9.2 ± 0.5 vs. 7.5 ± 0.5 s, $p = 0.029$; Fig. 2a), time from 1/2 peak to peak of the wash-in phase (4.4 ± 0.3 vs. 3.1 ± 0.3 s, $p = 0.0072$; Fig. 2b), and time from peak to 1/2 peak of the wash-out phase (5.6 ± 0.3 vs. 3.0 ± 0.3 s, $p < 0.0001$; Fig. 2d). However, no difference in the peak intensity was observed between the LC and control groups (2.1 ± 0.3 vs. 2.5 ± 0.3 s, $p = 0.43$; Fig. 2c).

Correlation of outflow times on CE-US and ΔCPR

The ΔCPR in the LC group was significantly lower than that in the control group (2.3 ± 0.3 vs. 3.7 ± 0.3 ng/ml, $p = 0.0050$; Fig. 2e). The drainage times expressed as the time from peak to 1/2 peak showed a significant correlation with ΔCPR ($R = 0.42$, $p = 0.0069$; Fig. 2f).

Pancreatic vessels

The wall thickness of the pancreatic vein was typically greater in the LC group (Fig. 3a) than in the control group (Fig. 3b) (major axis: 40.2 ± 3.8 vs. 22.3 ± 3.5 μm, $p = 0.0014$; minor axis: 27.0 ± 3.1 vs. 13.6 ± 2.8 μm, $p = 0.0025$; Fig. 3c, d).

Picrosirius Red staining revealed a substantial amount of collagen in the pancreatic vessel walls (primarily the intima) in the LC group (Fig. 3e). As shown by polarization microscopy, collagen type 3 was observed primarily at these intensely stained sites. A similar pattern of collagen deposition was not evident in the control group (Fig. 3e–h).

Islets and immunohistochemistry

Islet hypertrophy and a decrease in the percentage of insulin-positive area per islet were observed in the tissues of the LC group (Fig. 4a, b). The diameters of the islet cells were significantly increased in the LC group, as compared with the diameters in the control group (major axis: 320.8 ± 11.0 vs. 141.5 ± 10.2 μm, $p < 0.0001$, minor

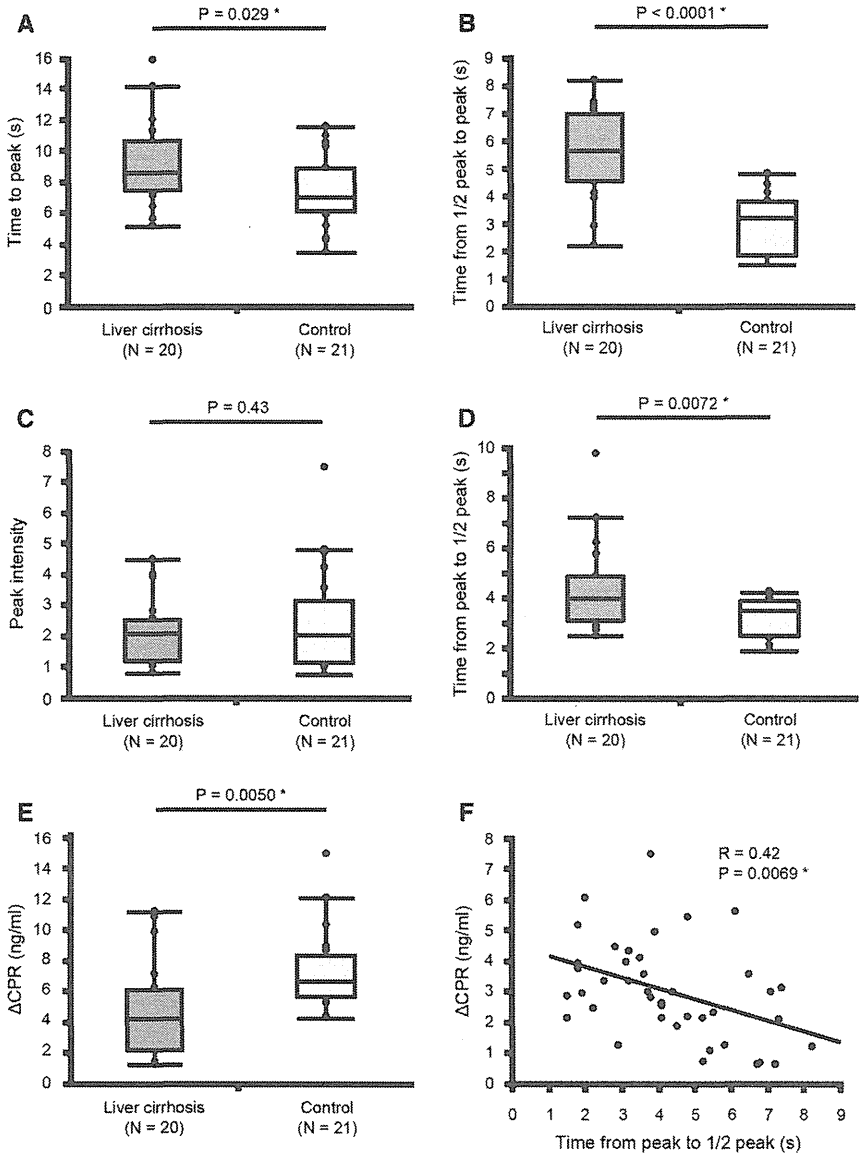

Fig. 2 Analyses of the CE-US time–intensity curves for pancreatic blood flow and ΔCPR. The *tops and bottoms of the boxes* indicate the first and third quartiles, respectively. The box length represents the interquartile range, with 50 % of the values located within it. **a** Time to peak, **b** time from 1/2 peak to peak of the wash-in phase, **c** peak intensity, **d** time from peak to 1/2 peak of the wash-out phase, and **e** ΔCPR. **f** A significant correlation was observed between the drainage time (expressed as the time from peak to 1/2 peak of the washout phase) and the ΔCPR ($R = 0.42$, $p = 0.0069$). *CE-US* contrast-enhanced ultrasound, *ΔCPR*, delta C-peptide immunoreactivity

axis: 236.0 ± 8.9 vs. 99.6 ± 8.3 μm, $p < 0.0001$, Fig. 4c, d). Although the number of insulin-positive cells did not differ between the LC and control groups (31.6 ± 3.3 vs. 40.4 ± 3.0, $p = 0.053$, Fig. 4e), the percentage of insulin-positive cells per islet was significantly lower in the LC group than in the control group (50.3 ± 2.8 vs. 66.6 ± 2.6 %, $p = 0.0001$, Fig. 4f). Moreover, the percentage of insulin-positive area per islet was significantly lower in the LC group, as compared with the control group (45.8 ± 2.2 vs. 75.5 ± 2.1 %, $p < 0.0001$, Fig. 4g). Additionally, the percentage of insulin-positive area per islet was significantly correlated with the wall thickness of the pancreatic vein($R = 0.63$, $p < 0.0001$, Fig. 4h). However, no difference between the LC and control groups was observed in terms of the percentage of glucagon-positive area per islet (26.0 ± 2.1 vs. 26.6 ± 2.0 %, $p = 0.85$).

Discussion

In this study, we found that the pancreas in LC patients showed congestive changes as assessed by CE-US images and histological findings. Further, we determined that the pancreatic congestive changes were significantly correlated with reduced insulin secretion.

As has been observed in previous reports, the typical tissue–intensity curve for the pancreas following bolus injection showed a rapid increase followed by a short peak

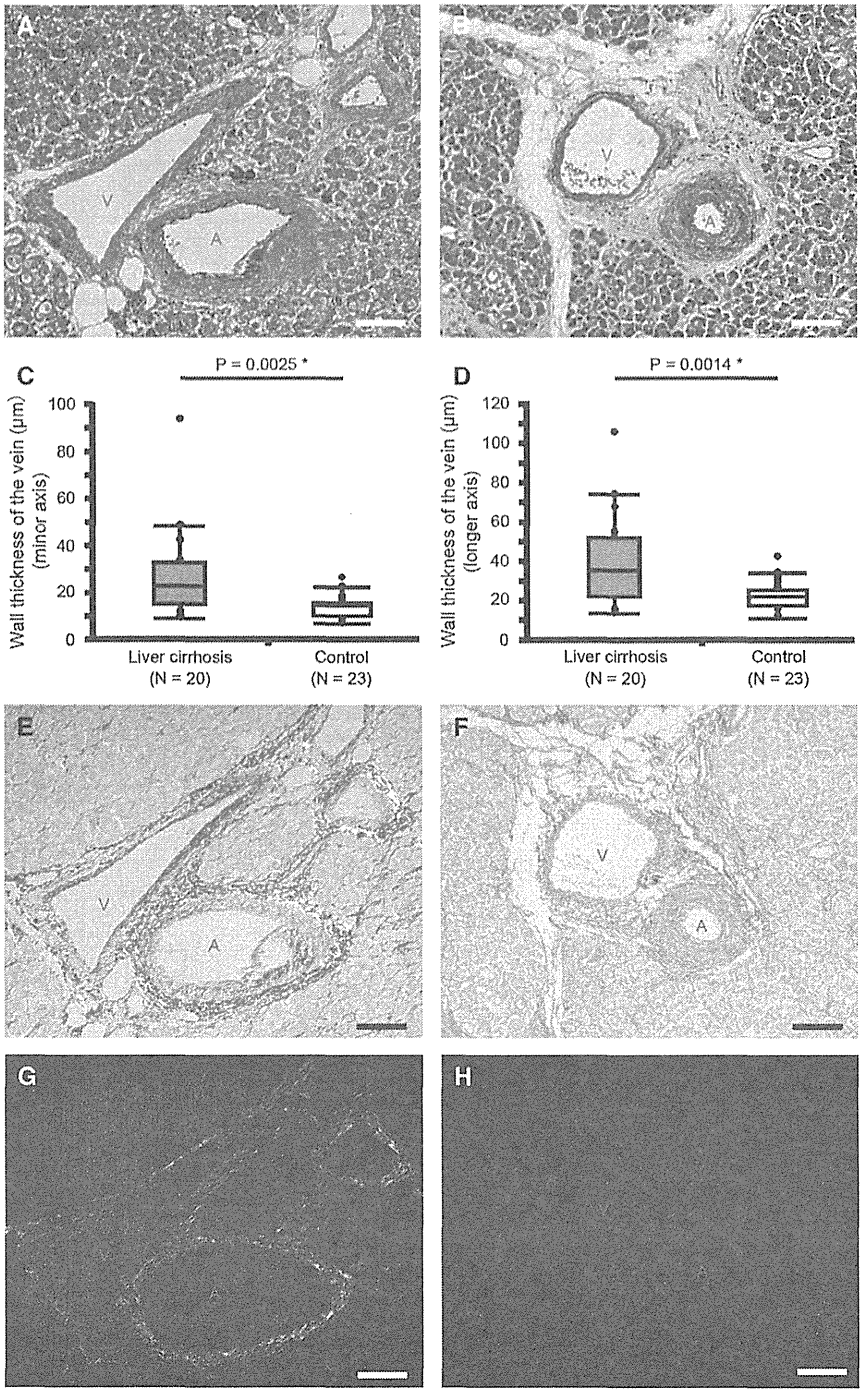

Fig. 3 Analyses of the pancreatic vein wall. **a**, **b** Elastica–Masson staining revealed that the wall thickness of the pancreatic vein was greater in the LC group (**a**) than in the control group (**b**). **c**, **d** The wall thickness of the pancreatic vein for the minor (**c**) and major axes (**d**). **e**, **f** As shown by Picrosirius Red staining, a substantial amount of collagen (*brilliant red*) was present in vessel walls in the LC group (**e**), as compared with the control group (**f**). **g**, **h** As revealed by polarization microscopy, collagen type 3 (*green*) primarily coincided with the areas that were stained most intensely by Picrosirius Red. The *yellow* coloration indicates collagen type 1 (**g**). This pattern was not evident in the control group (**h**). *LC* liver cirrhosis, *V* vein, *A* artery. *Scale bar* 100 μm

of strong enhancement with a fast washout [18, 19]. Because of this pronounced pattern, CE-US is widely applied for detecting and characterizing pancreatic disease in humans (e.g., pancreatic adenocarcinoma and acute pancreatitis) [20, 21]. However, no previous study has evaluated portal hypertension using CE-US. Here, we found that LC patients strongly suspected of having portal hypertension had a drainage disorder with respect to pancreatic blood flow.

In the present study, pathological changes in the pancreas due to portal hypertension were also observed (Figs. 3a, 4a), and these data support the findings of a drainage disorder in pancreatic blood flow on CE-US. The vessel walls of various organs (e.g., the spleen and lung)

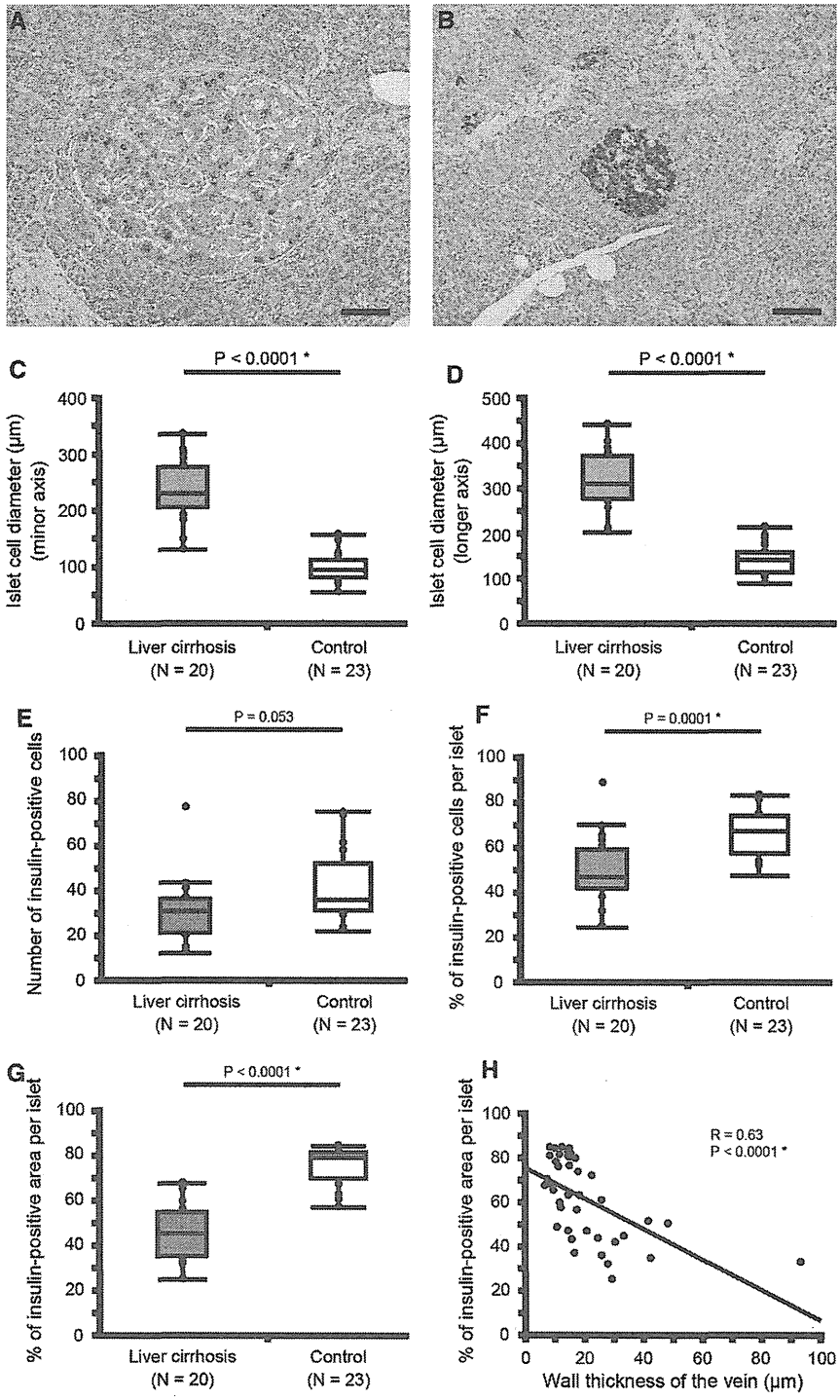

Fig. 4 Analyses of islet diameters and the percentage of insulin-positive areas. **a**, **b** Insulin staining of the islets by immunohistochemistry. Islet hypertrophy and a decrease in insulin-positive cells (brown staining) were observed in the LC group (**a**), as compared with the control group (**b**). **c**, **d** Diameters of the islet cells for the minor (**c**) and major (**d**) axes. **e** Percentage of insulin-positive areas within the islets. **f** A significant correlation was observed between the percentage of insulin-positive areas and the wall thickness of the pancreatic vein ($R = 0.63$, $p < 0.0001$). *LC* liver cirrhosis, *V* vein, *A* artery. *Scale bar* 100 μm

become thicker as an adaptive compensatory response to increased pressure from portal hypertension [22–25]. Li et al. [22] showed that collagenous fibers along splenic venous walls were increased, and that the expression intensity of type 3 procollagen mRNA was significantly higher in the splenic venous walls of patients with portal hypertension, as compared with controls. In our study, the pancreatic vein wall of LC patients showed thickening, and a substantial amount of collagen, particularly type 3 collagen, was deposited in the venous walls. These histological changes suggest that profound damage occurred to the intima of the pancreatic vessels in patients with portal

hypertension and could have resulted in impaired drainage blood flow.

Hyperinsulinemia and insulin resistance have been suggested to underlie the pathogenesis in hepatogenous diabetes, since intrahepatic and extrahepatic portosystemic shunts may contribute to decreased hepatic insulin clearance [4, 5]. Indeed, B-RTO resulted in an improvement in hepatic function accompanied by an increased portal venous flow, leading to increased degradation of insulin by the liver because of ameliorated hepatic parenchymal function [26–29]. However, in the present study, pancreatic congestive changes showed a significant correlation with a decrease in insulin secretion in both the clinical and pathological studies. Based on these findings, we believe that the impaired glucose tolerance in LC patients not only results from decreases in insulin clearance in the liver, but also results from a disorder in β-cell function (S2). Previous research using rats showed that the insulin secretion of pancreatic islets decreased in LC against a background of hyperinsulinemia [6, 7]. In addition, Narita et al. [30] demonstrated that total insulin secretion did not differ significantly between patients with chronic hepatitis C and subjects with normal glucose tolerance, whereas early phase insulin secretion in patients with chronic hepatitis C was lower than that in subjects with normal glucose tolerance. They concluded that the transition from normal to impaired glucose tolerance was accompanied by progressive deterioration of the early-phase insulin secretion rather than insulin resistance. In our study, ΔCPR was significantly lower in the LC group than in the control group, but basal insulin secretion was preserved. These findings are consistent with the previous results noted above. Further, our findings are consistent with the clinical condition of hepatogenous diabetes, as characterized by postprandial hyperglycemia and fasting hypoglycemia.

Although we did not identify the mechanism by which congestion could cause the hypertrophy of islets and the impairment of insulin secretion, some prior studies provide relevant evidence. Brüning et al. [31] suggested that the disruption of insulin signaling in the liver induces β-cell hyperplasia. Imai et al. [32] suggested that hepatic activation of extracellular regulated kinase (ERK) signaling induces pancreatic β-cell proliferation through a neuronal-mediated relay of metabolic signals. In patients with hepatitis C, the production of cytokines during hepatitis C virus-induced inflammatory processes in liver tissue could explain the progressive impairment of β-cell function with increasing liver damage [33]. Taken together, it is conceivable that decreased portal flow and congestion-caused inflammation in the liver may result in the hepatic activation of some signals, followed by the hypertrophy of islets and the impairment of insulin secretion.

Our study has several limitations. First, because we did not measure the hepatic venous pressure gradient, there was no evidence that all of the patients in the LC group had portal hypertension. However, the patients in the LC group had significant clinical parameters indicative of portal hypertension (Table 1). Second, we were unable to obtain portal blood, which might have enabled pancreatic insulin secretion to be determined directly. However, obtaining portal samples can be highly invasive and could therefore raise ethical issues. To overcome this dilemma, we performed the glucagon challenge test, which can also avoid problems with digestion and absorption in patients with LC [34–37]. Sherwin et al. [38] have reported that LC patients generally show hyperglucagonemia; therefore, sensitivity to glucagon might have decreased in the LC group in the present study. However, Sherwin et al. also found that the plasma glucagon concentration was elevated in LC patients with spontaneous portal systemic shunting, but was comparable for LC patients without portal systemic shunting and controls. Our study did not include basal glucagon data, but did include the basal C-peptide/insulin molar ratio, which suggests the presence of portal systemic shunts. This ratio did not differ between the two groups (Table 1), perhaps supporting the idea that basal glucagon does not differ substantially between the two groups. Third, because populations differed between the CE-US and pathological studies, this study could not prove whether the pancreases in the former study had findings of pathological congestion. Although endoscopic ultrasound fine-needle aspiration is available as an effective method for obtaining pancreatic tissues in vivo, it is difficult to obtain enough pancreas tissue for evaluation. To exclude this bias, we used tissue obtained from patients with or without LC at autopsy.

In conclusion, the presence of pancreatic congestion was evident both clinically and pathologically in LC patients. Further, pancreatic congestion correlated with decreased insulin secretion of the pancreas. Thus, in LC patients, portal hypertension might be among the factors that induce hepatogenous diabetes. If so, the treatment of portal hypertension in LC patients, including B-RTO, splenectomy, or β-blockers, may have the potential to improve hepatogenous diabetes. Further clinical trials are required to address these different possibilities.

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Conflict of interest The authors declare that they have no conflicts of interest.

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Prognosis and therapy for ruptured hepatocellular carcinoma: Problems with staging and treatment strategy

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ABSTRACT

Background: There are no clear criteria established for treating a ruptured hepatocellular carcinoma (HCC). To elucidate the clinical features of affected patients, we examined prognosis and therapy choices.
Materials/methods: We enrolled 67 patients treated for a ruptured HCC (HCV 44, HBV 5, HBV+HCV 1, alcohol 2, others 15; naïve HCC 34, recurrent 33) from 2000 to 2013, and investigated their clinical background and prognosis.
Results: Median survival time (MST) for all cases was 4 months. For patients who survived for more than 1 year after rupture, the percentages of Child-Pugh C and positive for portal vein tumor thrombosis (PVTT)/extrahepatic metastasis were less than for those who died within 1 year. Child-Pugh classification (A:B:C = 14:15:5 vs. 4:9:20, $P < 0.001$) was better, while the percentage of patients with multiple tumors was lower [19/34 (55.9%) vs. 29/33 (87.9%), respectively; $P < 0.001$] in the naïve group. The 1- and 3-year survival rates were better in the naïve as compared to the recurrent group (60.6% and 33.3% vs. 12.6% and 0%, respectively; $P < 0.01$). MST according to modified TNM stage (UICC 7th) calculated after exclusion of T4 factor of rupture, stage I was better than others (22.7 vs. (II) 2.2, (III) 1.2, and (IV) 0.7 months) ($P \approx 0.010$).
Conclusion: In patients with a ruptured HCC, especially those with a single tumor, and without decompensated liver cirrhosis and PVTT/extrahepatic metastasis, better prognosis can be expected with curative treatment. The present naïve group included more of such cases than the recurrent group, indicating the effectiveness of curative therapy.

1. Introduction

Some patients are diagnosed with hepatocellular carcinoma (HCC) following rupture as the initial symptom. In addition, repeated recurrence and progression are often observed, with rupture occurring in some of those cases.

A patient with a ruptured HCC is generally considered to have a poor prognosis and treated as T4 in the 7th edition of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) [1]. In the AJCC/UICC TNM system, T4 is defined as "tumors with direct invasion of adjacent organs other than the gallbladder, or perforation of visceral peritoneum", thus rupture of an HCC is classified as T4. Patient prognosis following rupture of an HCC is generally poor and the mortality rate of acute phase cases has been reported to range from 25% to 75% [2]. However, some affected patients show a good clinical course. In this context, it may be problematic that all ruptured HCC cases are classified as T4. Furthermore, among the various guidelines for treatment of an HCC, there is no clear consensus regarding how to treat a patient following rupture.

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We retrospectively evaluated the clinical features of HCC rupture cases, as well as therapy and prognosis to clarify the best treatment for affected patients.

2. Materials/methods

From January 2000 to December 2013, total 1460 patients with HCC (naïve HCC 1252, recurrence with past history of treatment 208) (total 4182 admissions) of Ehime Prefectural Central hospital were enrolled. Of those, 67 patients treated for a ruptured HCC (71 ruptures) were enrolled (HCV 44, HBV 5, HBV+HCV 1, alcohol 2, others 15). There were 34 patients without a past history of HCC (naïve group) and 33 recurrent patients (recurrence group). We investigated their clinical backgrounds and prognoses of patients who died within 3 months and of those who died within 1 year after rupture, retrospectively. In addition, we examined factors in patients with a good prognosis and clinical course after rupture. We modified TNM stage (UICC 7th) and used it for evaluation of the prognosis by tumor status after excluded T4 factor of rupture (modified TNM stage).

The diagnosis of HCC was based on past pathological findings, or evidence of tumor formation in the liver (with arterial hypervascularization) shown by dynamic computed tomography (CT) imaging [3] and/or angiography.

Transcatheter arterial embolization (TAE) was performed in cases considered to be tolerant of that treatment and without severely poor hepatic reserve function (total bilirubin over 3 mg/dL) after improvement from hypovolemic shock status. Radiologists and hepatologists performed all of the TAE procedures. A microcatheter was inserted into the artery feeding the ruptured tumor after a conventional hepatic angiography examination. A gelatin sponge (Gelform®, Upjohn, Kalamazoo, MI, USA; or Gelpart®, Nippon Kayaku Co., Ltd., Tokyo, Japan) was used for embolization. The goal of embolization was disappearance of staining of the ruptured tumor. After stabilization of systemic circulation and/or stopping of bleeding in cases with active bleeding upon admittance, surgical resection was planned from 2 weeks to 1 month after the rupture event, especially in cases with good hepatic reserve function and whose tumor(s) were considered to be resectable.

The present study permitted by ethics committee of Ehime Prefectural Central hospital. The authors have no financial conflicts of interest to disclose concerning this study.

2.1. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Student's *t* test for unpaired data, Fischer's exact test, a Mann-Whitney U test, and a log-rank test, as appropriate. All statistical analyses were performed using SPSS 21 J (SPSS Japan Inc., Tokyo, Japan), with a *p* value less than 0.05 considered to show statistical significance.

3. Results

Of a total 4182 admissions, 71 were ruptured HCCs (1.7%). A rupture occurred in 2.7% of naïve cases (34/1252) and 4.6% of all patients (67/1460). Median survival time (MST) for all cases was 4 months. Twenty-one of the 67 (31.3%) patients with rupture died within 1 month after the event (naïve group 5, recurrence group 16) and 30 (44.8%) died within 3 months. The 1-, 3-, and 6-month, and 1- and 2-year survival rates were 64.1%, 52.0%, 44.7%, 36.7%, and 22.9%, respectively (Fig. 1).

We analyzed the clinical features and therapeutic choices of patients who survived for more than 3 months after rupture ($n=30$) and those who died within 3 months ($n=30$) after exclusion of patients who were alive and whose observation period was under 3 months ($n=7$). Total-bilirubin and Child-Pugh class were better, and the frequencies of single tumor and naive status was greater in those who survived more than 3 months after rupture as compared to those who died within 3 months (Table 1).

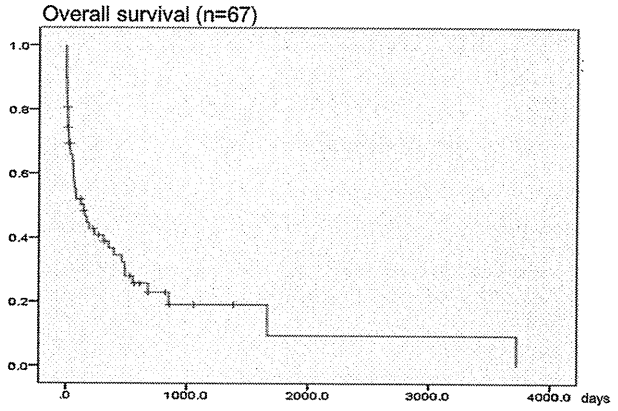

Fig. 1. Survival rates of patients with ruptured HCC ($n=67$).
The 1-, 3-, and 6-month, and 1- and 2-year survival rates were 64.1%, 52.0%, 44.7%, 36.7%, and 22.9%, respectively.

We analyzed the clinical features and therapeutic choices of those who survived over 1 year after rupture ($n=17$) and compared them to those who died within 1 year ($n=38$) after exclusion of patients who were alive and whose observation period was under 1 year ($n=12$). Among those who were alive over 1 year after rupture, total-bilirubin and albumin levels, and Child-Pugh class were better, while the frequencies of single tumor, negative for portal vein tumor thrombosis (PVTT) and/or extrahepatic metastasis, and naïve status were greater than those of patients who died within 1 year (Table 2).

When TNM stage (UICC 7th) was calculated after exclusion of the T4 factor of rupture (modified TNM), MST for modified TNM stage I was better than that for the other stages (20.7 vs. (II) 2.2, (III) 1.2, and (IV) 0.7 months, respectively; $P=0.009$) (Fig. 2).

Clinical data for the naïve and recurrence groups are shown in Table 3. The levels of albumin (2.59 ± 0.69 vs. 3.33 ± 0.50 g/dL, $P=0.021$) and fucosylated alpha-fetoprotein (31.7 ± 27.1 vs. 31.1 ± 33.5, $P=0.041$), and Child-Pugh classification (A:B:C = 4:9:20 vs. 14:15:5, $P<0.001$) were worse, while the percentage of patients with multiple tumors was greater [29/33 (87.9%) vs. 19/34 (55.9%), $P<0.001$] in the recurrence group than the naïve group. There were no significant differences between the groups in regard to other

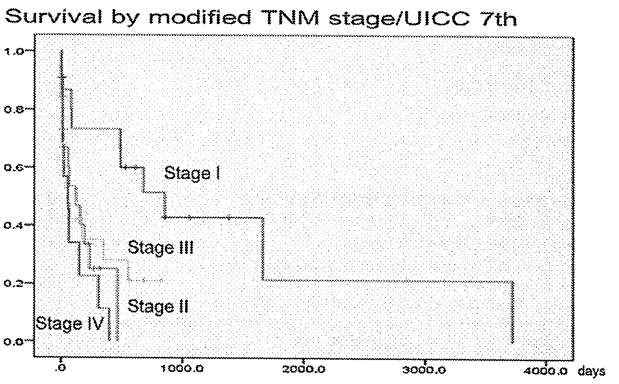

Fig. 2. Survival rates according to modified TNM stage (UICC 7th) calculated after exclusion of T4 factor. The median survival times for modified stage I, II, III, and IV were 20.7, 2.2, 1.2, and 0.7 months, respectively ($P=0.010$).

Table 1
Clinical features of HCC rupture patients who died within 3 months after rupture and survived for more than 3 months.

	Died within 3 months (n = 30)	Alive over 3 months (n = 30)	P value
Age (yrs)	70.1 ± 10.8	68.0 ± 11.9	0.458[a]
Sex (M:F)	23:7	28:2	0.039[b]
Etiology HCV:HBV:HBV&HCV:Alcohol:others	22:2:0:0:6	18:3:1:1:7	0.169[c]
Platelets (×10^4 cells/μL)	15.8 ± 8.4	15.8 ± 8.0	0.536[a]
AST (IU/L)	295.6 ± 848.4	98.6 ± 7.90	0.048[a]
ALT (IU/L)	64.9 ± 60.8	67.7 ± 51.2	0.618[a]
T-Bilirubin (mg/dL)	2.80 ± 1.84	1.13 ± 0.71	<0.001[a]
Albumin (g/dL)	2.56 ± 0.64	3.32 ± 0.55	0.152[a]
Prothrombin time (%)	59.1 ± 24.8	78.9 ± 19.2	0.271[a]
Child-Pugh Stage (A:B:C)	2:8:20	14:13:3	<0.001[c]
AFP (ng/mL)	118585.4 ± 262483.0	3723.1 ± 12162.3	<0.001[a]
AFP-L3 (%)	46.7 ± 29.3	16.1 ± 26.3	0.247[a]
DCP (mAU/mL)	31789.5 ± 29816.9	16951.0 ± 26837.4	0.161[a]
Average maximum tumor diameter (cm) (range)	7.45 ± 3.63 (3.1 to 14.0)	6.96 ± 4.35 (1.5 to 16.0)	0.412[a]
Tumor number	4.07 ± 2.16	2.53 ± 1.94	0.141[a]
Tumor number (single:multiple)	6:24	16:14	0.008[b]
Positive for extrahepatic metastasis and/or PVTT	12 (40.0%)	7 (23.3%)	0.169[b]
naïve:recurrence	10:20	20:10	0.010[b]
Treatment (Ope:RFA:OPE&RFA:TAE:others:BSC)	2:0:0:7:1:20	12:1:3:11:2:1	<0.001[c]

[a] Student's t test for unpaired data.
[b] Fischer's exact test.
[c] a Mann-Whitney U test.
HCV: hepatitis C virus, HBV: hepatitis B virus, AST: aspartate aminotransferase, ALT: alanine aminotransferase, AFP: alpha-fetoprotein, AFP-L3: fucosylated AFP, DCP: des-gamma carboxy prothrombin, PVTT: portal vein tumor thrombosis, Ope: surgical resection, RFA: radiofrequency ablation therapy, TAE: transcatheter arterial embolization, BSC: best supportive care

clinical background factors including age, sex, and etiology. In the naïve group (n = 34), surgical resection was performed in 11 (32.4%), collaborated therapy resection and RFA in 3 (8.8%), and RFA in 1 (2.9%) as curative treatment, while surgical resection as curative treatment was performed in only 1 (3.0%) in the recurrence group (n = 33). Of all 16 patients treated curatively, TAE was performed during the acute phase of rupture for stopping bleeding and stabilization of systemic circulation in 81.3% (13/16). A representative case is shown (Fig. 3). There were another 3 patients who were treated with resection as mass reduction surgery for preventing re-rupture (naïve 2, recurrence 1). MST was better in patients treated curatively (n = 16) as compared to those treated non-curatively (n = 51) (22.7 vs. 1.8 months, P < 0.001). The 1-, 5-, and 10-year survival rates after curative treatments were 79.4%, 49.0% and 49.0%, respectively (Fig. 4a). The 6-month, and 1- and 3-year survival rates were better in the naïve group than recurrent group (60.6%, 60.6%, and 33.3% vs. 31.5%, 12.6%, and 0%, respectively; P < 0.001) (Fig. 4b). Moreover, the survival rates of patients without PVTT and/or extrahepatic metastasis (n = 20) were better than those of patients with those factors (n = 14) in the naïve group (83.8%, 83.8%, and 49.6% vs. 16.2%, 0%, and 0%, respectively; P < 0.001) (Fig. 5).

Among patients who were observed for more than 1 month after rupture in both group (n = 39), peritoneum dissemination was noted in 3 (7.7%) at 1, 3, and 5 months. There were 4 cases of re-rupture that occurred more than 1 month after the first rupture (naïve group 1 vs. recurrence group 3). The recurrence group had a higher risk ratio for death as compared to the naïve group (HR 3.14, 95%CI: 1.68–5.84; P < 0.001).

Table 2
Clinical features of HCC patients who died within 1 year after rupture and survived for more than 1 year.

	Died within 1 year (n = 38)	Alive over 1 year (n = 17)	P value
Age (yrs)	68.9 ± 11.0	68.8 ± 11.7	0.661[a]
Sex (M:F)	29:9	16:1	0.117[b]
Etiology HCV:HBV:HBV&HCV:Alcohol:others	26:4:0:0:8	11:0:0:2:4	0.641[c]
Platelets (×10^4 cells/μL)	15.1 ± 8.7	15.8 ± 4.6	0.010[a]
AST (IU/L)	251.9 ± 756.5	107.2 ± 94.6	0.228[a]
ALT (IU/L)	60.9 ± 55.9	74.1 ± 55.4	0.878[a]
T-Bilirubin (mg/dL)	2.53 ± 1.76	1.04 ± 0.64	<0.001[a]
Albumin (g/dL)	2.68 ± 0.71	3.39 ± 0.39	0.002[a]
Prothrombin time (%)	61.3 ± 24.1	80.0 ± 16.7	0.111[a]
Child-Pugh Stage (A:B:C)	5:10:23	9:8:0	<0.001[c]
AFP (ng/mL)	92623.9 ± 232284.0	13897.2 ± 23477.8	0.003[a]
AFP-L3 (%)	39.8 ± 31.9	11.8 ± 21.3	0.005[a]
DCP (mAU/mL)	29628.0 ± 30267.3	13897.2 ± 23477.8	0.019[a]
Average maximum tumor diameter (cm) (range)	7.13 ± 4.12 (1.5-16.0)	7.19 ± 3.65 (2.5 to 15.0)	0.286[a]
Tumor number	3.89 ± 2.15	2.12 ± 1.87	0.031[a]
Tumor number (single:multiple)	8:30	12:5	<0.001[b]
Positive for extrahepatic metastasis and/or PVTT	16 (42.1%)	1 (5.9%)	<0.001[b]
naïve:recurrence	12:26	14:3	0.001[b]
Treatment (Ope:RFA:OPE&RFA:TAE:others:BSC)	3:0:0:11:3:21	8:1:3:5:0:0	<0.001[c]

[a] Student's t test for unpaired data.
[b] Fischer's exact test.
[c] a Mann-Whitney U test.
HCV: hepatitis C virus, HBV: hepatitis B virus, AST: aspartate aminotransferase, ALT: alanine aminotransferase, AFP: alpha-fetoprotein, AFP-L3: fucosylated AFP, DCP: des-gamma carboxy prothrombin, PVTT: portal vein tumor thrombosis, Ope: surgical resection, RFA: radiofrequency ablation therapy, TAE: transcatheter arterial embolization, BSC: best supportive care

Table 3
Clinical background of naïve and recurrent cases.

	Naïve ($n=34$)	Recurrence ($n=33$)	P value
Age (yrs)	68.9 ± 10.9	69.5 ± 11.5	0.660[a]
Sex (M:F)	31:3	26:7	0.158[b]
HCV:HBV: HBV&HCV:Alcohol:others	20:2:0:2:10	24:3:1:0:5	0.174[c]
Platelets ($\times 10^4$ cells/μL)	17.8 ± 8.0	12.6 ± 7.0	0.710[a]
AST (IU/L)	138.3 ± 167.5	232.0 ± 803.5	0.181[a]
ALT (IU/L)	80.2 ± 59.0	48.7 ± 43.5	0.066[a]
T-Bilirubin (mg/dL)	1.49 ± 1.38	2.38 ± 0.69	0.129[a]
Albumin (g/dL)	3.33 ± 0.50	2.59 ± 0.69	0.021[a]
Prothrombin time (%)	76.2 ± 22.3	60.6 ± 22.0	0.750[a]
Child-Pugh Stage (A:B:C)	14:15:5	4:9:20	<0.001[c]
AFP (ng/mL)	46317.0 ± 191159.2	55466.0 ± 159112.2	0.844[a]
AFP-L3 (%)	31.1 ± 33.5	31.7 ± 27.1	0.041[a]
DCP (mAU/mL)	20705.4 ± 26461.5	27680.1 ± 31759.3	0.058[a]
Average maximum tumour diameter (cm) (range)	8.5 ± 4.1 (2.0-16.0)	5.9 ± 3.6 (1.5-15.0)	0.291[a]
Tumor number (single:multiple)	17:17	4:29	<0.001[b]
Positive for extrahepatic metastasis and/or PVTT	14 (41.1%)	8 (24.2%)	0.143[b]
Treatment (Ope:RFA::OPE&RFA:TAE:others:BSC)	13:1:3:8:2:7	2:0:0:14:2:15	0.001[c]
Death within 1/3 months	5 (14.7%)/10 (29.4%)	16 (48.5%)/20 (60.6%)	0.002[b]/0.010[b]
Multiple rupture events in cases observed over 1 month	1 (4.0%; 1/25)	3 (21.4%: 3/14)	0.016[b]

[a] Student's t test for unpaired data.
[b] Fischer's exact test.
[c] a Mann-Whitney U test.
HCV: hepatitis C virus, HBV: hepatitis B virus, AST: aspartate aminotransferase, ALT: alanine aminotransferase, AFP: alpha-fetoprotein, AFP-L3: fucosylated AFP, DCP: des-gamma carboxy prothrombin, PVTT: portal vein tumor thrombosis, Ope: surgical resection, RFA: radiofrequency ablation therapy, TAE: transcatheter arterial embolization, BSC: best supportive care

4. Discussion

Because of development of surveillance and imaging modalities for HCC, the ratio of rupture has become smaller recently (2.7% of naïve HCC and 4.6% of all patients in the present study) than the past reports in Japan (10% in the 1990s [4] and 7.4% in the 2000s [5]). An HCC rupture is thought to be a life-threatening event, as it has a hypervascular characteristic and a rupture can easily lead to development of hypovolemic status. The mortality rate associated with a ruptured HCC in the acute phase has been reported to range from 25% to 75% [2]. Furthermore, the 1-month survival rate of patients with a ruptured HCC has been found to range from 35% to 67% [6,7]. Miyoshi et al. reported that the mortality rate with 1 year was 40% and MST was 10 months [8]. In the present study, 31.3% (21/67) of our patients died within 1 month after the rupture event for a 1-month mortality rate of 35.9%, and the 1-year mortality rate was 63.3%. The patients who survived for more than 1 year had larger frequencies of naïve status, single tumor, Child-Pugh A or B, and negative for PVTT and extrahepatic metastasis as compared to those who died within 1 year after the rupture event. Also, the

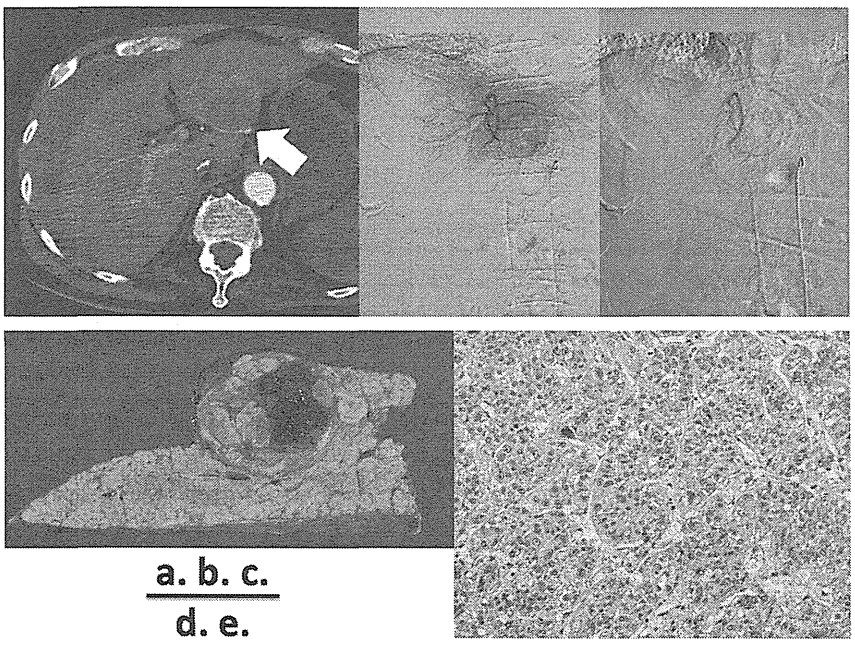

Fig. 3. An 84-years Japanese man, who had no viral hepatic disease, with ruptured hepatocellular carcinoma in left lobe (4.3 cm in diameter) (a. enhanced computed tomography, artery phase: arrow). One month after stabilized shock status with transcatheter arterial embolization (TAE) (b: pre-TAE) (c: post-TAE), surgical resection was performed (d). Moderate differentiated hepatocellular carcinoma (e) and liver cirrhosis was diagnosed pathologically, and he is alive without peritoneal metastasis 4 years after rupture.

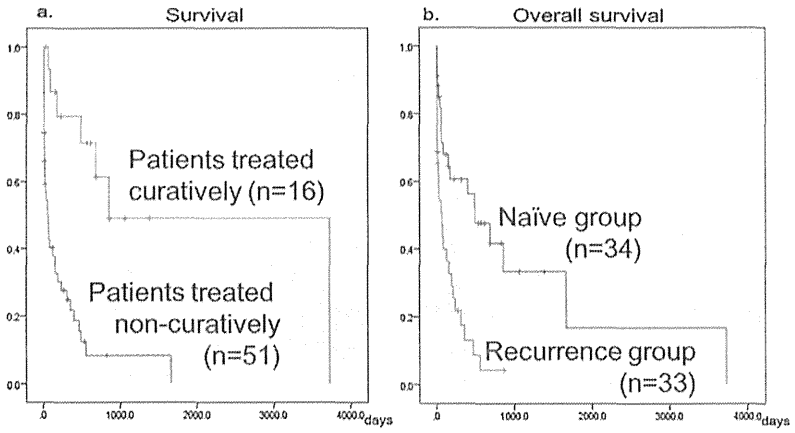

Fig. 4. a. Survival rates of patients treated curatively and non-curatively. Median survival time was better in those treated curatively ($n = 16$) as compared to non-curatively ($n = 51$) (22.7 vs. 1.8 months, $P < 0.001$). The 1-, 5-, and 10-year survival rates after curative treatments were 79.4%, 49.0% and 49.0%, respectively. b. Survival rates of naïve and recurrence groups. The 6-month, and 1- and 3-year survival rates of the naïve group ($n = 34$) were better than those of the recurrence group ($n = 33$) (60.6%, 60.6%, and 33.3% vs. 31.5%, 12.6%, and 0%, respectively; $P < 0.001$).

1-year survival rate of the naïve group was better than that of the recurrence group (60.6% vs. 12.6%). In analysis of TNM stage of UICC 7th, after excluding T4 as a factor (modified TNM stage), the MST of modified TNM stage I patients was better than that of those with other modified stages. The clinical impact of rupture was thought to differ with different background factors and the T4 factor of rupture cannot be indicated for all ruptured HCC cases.

It was reported that TAE for a ruptured HCC resulted in better prognosis than patients who underwent conservative treatment (MST: 110 vs. 9.0 days, $P < 0.003$) [6]. In our study, patients with a ruptured HCC treated curatively showed better prognosis than those not treated curatively (22.7 vs. 1.8 months, $P < 0.001$). The first step for treatment of a ruptured HCC is obtaining hemostasis and cardiorespiratory stabilization [9,10]. In addition, TAE can bridge some patients with a ruptured HCC to a hepatectomy procedure, while a majority of naïve cases can be treated by resection after TAE. In the present study, 81.3% (13/16) of our patients treated curatively with resection, collaboration of resection and RFA [11] or RFA had undergone TAE in the acute phase of rupture. The data of the 1-, 5- and 10-year survival rates after curative treatments of this study was similar to the past report by Yoshida which described that the 1-, 5-, and 10-year survival rates after an elective hepatectomy in patients with a ruptured HCC were 90.0%, 67.5% and 20.3%, respectively [9].

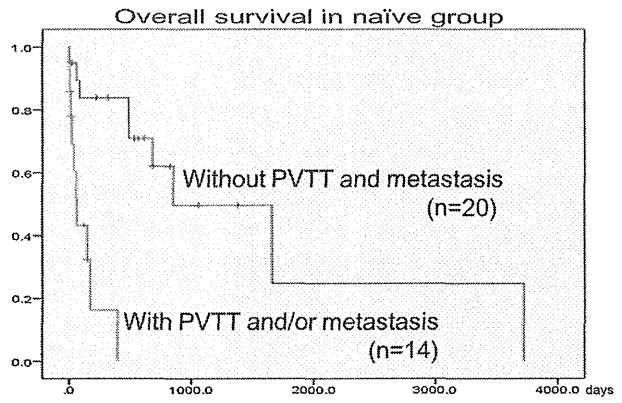

Fig. 5. Survival rates of naïve patients with and without portal vein tumor thrombosis (PVTT)/extrahepatic metastasis.
The 6-month, and 1- and 3-year survival rates of naïve cases without PVTT and metastasis ($n = 20$) were better as compared to those with PVTT and/or metastasis ($n = 14$) (83.8%, 83.8%, and 49.6% vs. 16.2%, 0%, and 0%, respectively; $P < 0.001$).

In the AJCC/UICC 7th TNM classifications, a ruptured HCC is classified as a T4 factor. In the American Association for the Study of Liver Disease (AASLD) practice guidelines for HCC [3], in which the Barcelona Clinic Liver Cancer (BCLC) staging system is used for treatment selection, as well as the Asian Pacific Association for the Study of the Liver (APASL) consensus recommendation [12], and the consensus-based clinical practice guidelines proposed by the Japan Society of Hepatology (JSH) [13], there are no clear indications for treatment of a ruptured HCC. In the BCLC staging system, which utilizes the AASLD practice guidelines, ruptured HCC has not been classified as any particular stage. In the various guidelines and algorithms available, included treatment options are mainly documented by the features of tumor size, number of tumors, hepatic reserve function, PVTT/extrahepatic metastasis, and performance status. In this context, it is important to determine an algorithm or guidelines for treatment of patients with a ruptured HCC in consideration of their background and current state. The prognosis of an affected patient is thought to be defined by hepatic reserve function prior to the rupture, total bleeding volume, and HCC progression including portal vein tumor thrombosis [14]. In the present study, prognoses differed between the recurrent and naïve cases, as the former showed a tendency of more advanced stage HCC and worse hepatic reserve function, which may influence prognosis. Of course, there was a selection bias between the patients who were treated curatively and the others. The patients who did undergo curative management must be with less advanced disease. However, there are some ruptured HCCs which show good prognosis by additional curative treatments. In principle, we should judge whether additional curative treatments can be performed or not, after stabilized general condition meticulously.

Furthermore, a ruptured HCC did not always show peritoneum dissemination. We found dissemination of HCC cells after rupture in only 7.7% of the present patients. Therefore, we recommend curative treatment for patients with a good clinical background.

Initial therapy with TAE to stabilize general condition is an important therapeutic step in many ruptured HCC patients with good hepatic reserve function. It was thought that curative treatment should be considered as a therapeutic option in patients with a ruptured HCC, especially in those with a single tumor and without PVTT/extrahepatic metastasis based on previous findings. In addition, T4 classification should not include all types of ruptured HCC.

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