Table 3 Grading system and representative management strategies for bile leakage

Grades	Definition	Management strategies
Grade A	No change in the patient's clinical management strategy required or manageable with simple drainage	Drainage within 7 d Antibiotic administration
Grade B	Manageable with interventional procedures	Drainage for 7 or more day, ethanol injection, fibrin paste injection, single ENBD, single EBD, single PTBD, PTPE, TAE
Grade C	Cases involving pneumoperitoneum, inflammation, multiple organ failure, or reoperation	Complicated IVR (combinations with any Grade Bs) Reoperation

ENBD: Endoscopic nasobiliary drainage; EBD: Andoscopic biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; PTPE: Percutaneous transcatheter portal embolization; TAE: Transcatheter arterial embolization; IVR: Interventional radiology.

Table 4 Grading system and representative management strategies for acute renal failure

Grades	Definition	Management strategies
Grade A	Increase in serum creatinine level of ≥ 0.3 mg/dL from the baseline or 1.5 to 2-fold increase from the baseline Urinary output of less than 0.5 mL/kg per hour for more than 6 h	Dehydration Diuretics
Grade B	Two-fold increase in the serum creatinine level from the baseline Urinary output of less than 0.5 mL/kg per hour for more than 12 h	Continuous mannitol + diuretics
Grade C	Dialysis treatment required (serum K > 6.0 mEq, BE < -10, uremia, hypouresis that lasts for more than three days)	Hemodialysis

Table 5 Grading system and representative management strategies for ascites

Grades	Definition in International Ascites Club (2003)	Definition in International Ascites Club (1996)
Grade A	Detected only on United States	Mild
Grade B	Moderate symmetrical distention of the abdomen	Moderate
Grade C	Marked abdominal distention	Massive or tense

Table 6 Grading system and representative management strategies for ascites

Grades	Definition	Management strategies
Grade A	Requiring any changes in the clinical management strategy or manageable with medication Ascites discharge < 1000 mL/d in the drainage case	Diuretics, sodium restriction
Grade B	Grade A ascites that lasts for more than 2 wk or requires peritoneal puncture Ascites discharge < 2000 mL/d in the drainage case	Peritoneal puncture
Grade C	Invasive treatment required	Denver peritoneovenous shunt, TIPS, PSE, splenectomy

TIPS: Transjugular intrahepatic portosystemic shunt; PSE: Partial splenic embolization.

focuses on decreasing the patient's portal pressure[27,28]. The use of diuretics or sodium restriction can decrease systemic flow volume, and ascites can also be controlled by decreasing edema in the inter-organ space or establishing a systemic shunt. Invasive management aims to decrease the patient's portal pressure through mechanical interventions. The IASC previously released statements containing revised definitions of ascites (Table 5); however, they were too abstract to use in academic studies. So, we proposed a modified grading system for post-operative ascites after liver resection (Table 6).

SURGICAL SITE INFECTIONS (SUPERFICIAL, ORGAN AND DEEP) AND WOUND DEHISCENCE

Surgical site infections (SSI) are common after all types of surgery and are classified into superficial, deep incisional, and organ/space SSI. Although several classifications of SSI have been proposed[30], the definitions developed by the Centers for Disease Control and Prevention (CDC) are widely used internationally[31]. According to the CDC, SSI are infections that occur within 30 d of surgery or within one year if an implant is present[31]. In addition, one of the following criteria must be met: (1) purulent drainage from an incision (incisional infection) or from a drain below the fascia (deep infection); (2) a surgeon or attending physician diagnosing an SSI; (3) an infective organism being isolated from a culture of fluid or tissue obtained from the surgical wound (for incisional infections); (4) spontaneous dehiscence or a surgeon deliberately re-opening the wound in the presence of fever or local pain, unless subsequent cultures were negative, or an abscess being detected during direct examinations (for deep infections). However, the grading of SSI based

Table 7 Grading system for superficial SSI and wound dehiscence

Grades	Definitions	Management strategies
Grade A	Manageable within 2 wk	Small open wound, outpatient service
Grade B	Requiring any management 2 wk and more	Large open wound, inpatient service
Grade C	Any management required under general anesthesia	

Table 8 Grading system for deep and organ/space surgical site infections

Grades	Definitions	Management strategies
Grade A	Manageable without requiring any additional perioperative management within 2 wk	Antibiotics, simple drainage
Grade B	Requiring any management 2 wk and more	Additional drainage, irrigation
Grade C	Any management required under general anesthesia	

Table 9 Grading system and representative management strategies for coagulation disorders

Grades	Definition	Managements
Grade A	Does not require any change in the clinical management strategy Plat < 10×10^4 (preoperative Plat was within normal range) 30% reduction in Plat (preoperative Plat was abnormal)	Vitamin K, ATIII, LMWH, SPI, UFH, and DS
Grade B	Medication required for more than 5 d Plat < 5×10^4 (preoperative Plat was within normal range) 60% reduction in Plat (preoperative Plat was abnormal)	Platelet transfusion
Grade C	Intensive care treatment required and involved the failure of other organs	

Plat: Platelet count; ATIII: Anti-thrombin; LMWH: Low molecular weight heparin; SPI: Synthetic protease inhibitor; UFH: Unfractionated heparin; DS: Danaparoid sodium.

Table 10 Grading system and representative management strategies for pneumonia and respiratory disorder

Grades	Definition	Managements
Grade A	Meet SIRS criteria with imaging findings in less than 50% of the lung field or PaO_2/FiO_2 < 300	Antibiotics and oxygen Sputum suction
Grade B	Meet SIRS criteria with imaging findings in 50% and more of the lung field or PaO_2/FiO_2 < 200	Antibiotics and oxygen, IPPV, NPPV, bronchoscopy for sputum suction
Grade C	Requiring ventilator support	Ventilator

Systemic inflammatory response syndrome criteria is defined as two or more of the following clinical signs: bodily temperature > 38 ℃ or < 36 ℃, heart rate > 90/min, respiratory rate > 20 /min or $PaCO_2$ < 32 mmHg, WBC > 12000/μL or < 4000 /μL or immature cells > 10%. Pneumonia imaging is any of air-space opacity, lobar consolidation, or interstitial opacities. SIRS: Systemic inflammatory response syndrome; IPPV: Intermittent positive-pressure breathing; NPPV: Nasal positive-pressure ventilation.

on symptoms and the management strategy employed is difficult. Therefore, we proposed that SSI should be graded based on how long they take to cure (Table 7 for superficial SSI and wound dehiscence, Table 8 for deep and organ/space SSI). Using this new grading system, it is very easy and simple to grade SSI objectively.

COAGULATION DISORDERS

Coagulation disorders are a common complication after liver resection[32,33]. Most coagulation and anti-coagulant factors are synthesized by the liver, and the ability to synthesize such factors rapidly deteriorates after liver resection in cirrhotic patients and those who experience marked hepatic volume loss[20]. In addition, most patients who are scheduled to undergo liver resection present with thrombocytopenia due to portal hypertension. Therefore, a prolonged prothrombin time, a prolonged thrombin time, elevated levels of fibrinogen degradation products, and a low platelet count are common after liver resection[34]. As we have mentioned in the ascites section, portal hypertension can occur after liver resection due to an increase in portal flow resistance[17]. Therefore, coagulation disorders should be divided into two different grades based on whether the patient displays normal or abnormal preoperative platelet levels (Table 9).

PNEUMONIA AND RESPIRATORY DISORDER

Postoperative pneumonia and respiratory disorder (PPN/RD) was rarely seen after liver resection recently except in the elderly cases[35,36]. Definition of the PPN/RD

was shown in Table 10. Clinical sign of the PPN/RD is systemic inflammatory response syndrome with any radiological imaging findings[37]. Management will be taken by administrating susceptible anti-biotics with oxygen supply. Acute lung injury (ALI) is defined by PaO_2/FiO_2 ratios < 300 and acute respiratory distress syndrome (ARDS) is defined by PaO_2/FiO_2 ratios < 200[38]. In our grading, ALI is in Grade A and ARDS is in the grade B (Table 10). Our grading is not only defined PPN/RD after liver resection but also after other general surgery.

CONCLUSION

The complications seen after liver resection are different from those encountered after other types of surgery because the liver produces most serum proteins, which play a major role in maintaining systemic homeostasis, and liver resection affects liver function. Therefore, post-liver resection complications tend to be severe. The risk factors for complications after liver resection depend on the pathological background of the liver itself[39]. In patients with normal liver function, the operative time, fresh frozen plasma transfusion requirement, tumor size, and retinol binding protein levels are independent risk factors for complications[40]. On the other hand, the PT and the indocyanine green retention value at 15 min are independent risk factors for complications in cirrhotic patients[40]. Therefore, consensus definitions and grading systems are necessary to allow comparisons between academic reports. Our grading system incorporates established consensus definitions and statements, such as those for PHLF and BL, and attempts to establish objective definitions for grading other complications. We hope that our grading system will be used to describe the complications experienced after liver resection.

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REVIEW ARTICLE

Preoperative liver function assessments to estimate the prognosis and safety of liver resections

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Abstract Liver function assessment is important to ensure safe surgical procedures in patients with hepatocellular disease. Because the liver influences a wide variety of functions, including protein synthesis and metabolic, immune and storage functions, no single parameter is sufficient to adequately address all of these functions. We reviewed the relevant literature concerning the scoring systems, functional tests, plasma parameters and imaging modalities currently used to evaluate the liver function in an attempt to determine which parameters provide the most comprehensive and useful results. While the Child–Pugh scoring system is the gold standard for liver disease assessment, the liver damage grading system recommended by the Liver Cancer Study Group of Japan is also useful. Various models for end-stage liver disease scoring are used for organ allocation. While the indocyanine green clearance test is widely accepted throughout the world, other assessments have not been used routinely for clinical evaluations. The levels of plasma proteins, including albumin, prealbumin, retinol binding protein, apolipoprotein, coagulation factors and antithrombin III, represent the liver productivity. Liver fibrotic markers also correlate with liver function. Imaging modalities such as ^{99m}Tc-galactosyl serum albumin scintigraphy, ^{99m}Tc-mebrofenin hepatobiliary scintigraphy and transient elastography are also available, but future studies are needed to validate their clinical efficacy.

Keywords Liver function · Hepatocellular carcinoma · Liver resection

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Introduction

Liver function assessment is important for estimating not only the prognosis, but also for determining the surgical indications in patients suffering from cirrhosis with or without cancer [1–5]. The Child–Pugh score is used worldwide for this purpose [6, 7]. However, this index is too simple to evaluate the liver function in detail, and is also inadequate to ensure the safety of surgical procedures. Therefore, various serum proteins have been measured as markers of liver function, and other metabolic tests have also been investigated (Table 1) [2, 4, 8]. The liver is involved in a wide variety of processes, including protein synthesis, metabolism, bile excretion and immune protection. Because of this wide variety of functions, no single parameter or test that measures all components simultaneously has been available to date. We herein present a review of the relevant literature concerning representative parameters, tests and measurements used for the assessment of liver function.

Child–Pugh score

The Child–Pugh scoring system (Table 2) includes assessment of the plasma albumin and bilirubin levels, prothrombin time and the presence or absence of ascites and hepatic encephalopathy [6, 7]. Albumin is the most

Table 1 Assessment of liver function: scoring, grading, tests, serum parameters, and imaging modalities

Liver function score or grade for total evaluation	
Child–Pugh score	
Liver damage grade as per the Liver Cancer Study Group of Japan (LSCGJ)	
The model for end-stage liver disease (MELD) score	
Assessment of metabolic function	
Indocyanine green clearance (ICG) test	
Monoethylglycinexylidide (MEGX) test	
Galactose elimination capacity test	
^{13}C-liver-function breath tests	
Assessment of protein synthesis	
Albumin	Coagulation factors
Prealbumin (transthyretin)	Retinol binding protein
Apolipoprotein	Antithrombin III
Assessment of liver fibrosis	
Aspartate aminotransferase-to-platelet ratio index (APRI)	
Hyaluronic acid (HA)	Collagens
Imaging modalities for assessment of liver function	
^{99m}Tc-galactosyl serum albumin scintigraphy	
^{99m}Tc-mebrofenin hepatobiliary scintigraphy	
Transient elastography (TE)	

Table 2 The Child–Pugh scoring system and the liver damage grading system

Parameters	Child–Pugh scores		
	1 point	2 points	3 points
Albumin (mg/dl)	>3.5	3.5–2.8	<2.8
Bilirubin (mg/dl)	<2	2–3	>3
PT-INR	<1.7	1.7–2.3	>2.3
PT (%)	>70	40–70	<40
Ascites	None	Small or controlled	Tense
Encephalopathy	Absent	State I or II	State III or IV

Class A: 5–6 total points, class B: 7–9 total points, class C: 10–15 total points

Parameters	Liver damage grades		
	A grade	B grade	C grade
Albumin (mg/dl)	>3.5	3.5–3.0	<3.0
Bilirubin (mg/dl)	<2	2–3	>3
PT (%)	>80	50–80	<50
Ascites	None	Small or controlled	Tense
$ICGR_{15}$ (%)	<15	15–40	>40

If the liver damage final grade meets more than one grade, then the worst grade should be adopted.

PT prothrombin green time, *INR* international ratio, $ICGR_{15}$ indocyanine green retention rate at 15 min

abundant protein in the human body, and is exclusively produced by hepatocytes. Its half-life is 14.8 days. The production of albumin is more stable than that of other proteins [9]. The assessment of the bilirubin level indirectly represents the uptake, conjugation and excretion of bile juice [10]. Therefore, the bilirubin level is a specific marker for serious liver injury and loss of function. However, many pathological conditions of the liver, which affect organic anion transporters, automatically alter bilirubin kinetics. Furthermore, the bilirubin level may also be increased by non-hepatic factors, such as hemolysis during sepsis. Therefore, the plasma bilirubin concentration does not always represent the liver function. Most coagulation factors are also produced by hepatocytes, and the

prothrombin time, including the international normalized ratio (INR), has been used as an indirect indicator of liver function [11]. A classification of grade A according to the patient's Child–Pugh scores is a typical indication for liver resection [1]. Alternatively, liver transplantation is selected if oncological indications meet the established criteria.

Liver damage grades recommended by the Liver Cancer Study Group of Japan (LCSGJ)

The liver damage grade classification proposed by the LCSGJ has been shown to be a better option for evaluating the functional reserve capacity of the liver (Table 2), especially in surgical candidates [12]. A major difference between the Child–Pugh scoring system and the liver damage grading system is that hepatic encephalopathy is excluded, while the indocyanine green retention rate at 15 min ($ICGR_{15}$) is included as a parameter in the latter. Minor differences between the two systems include the determination of the precise albumin levels (3.5–2.8 vs. 3.5–3.0 mg/dl, respectively) and prothrombin time (40–70 % vs. 50–80 %, respectively).

The model for end-stage liver disease (MELD) score

The MELD score is a mathematical equation used to allocate organs for liver transplantation [13–15]. Although the MELD score was initially validated for the prediction of short-term survival, it was also shown to be useful for the prediction of long-term survival in patients with cirrhosis [13]. A total of four independent prognostic predictors have been identified in the multivariate survival model. These predictors are the serum bilirubin level, serum creatinine level, INR of the prothrombin time and the etiology of cirrhosis (alcoholic and cholestatic vs. others). After modification by the United Network for Organ Sharing, the MELD equation currently used to calculate the severity score is as follows: 9.57 X ln (creatinine, mg/dl) + 3.78 X ln (bilirubin, mg/dl) + 11.2 X ln (INR) + 6.43. Various modified MELD formulas (Table 3) have been proposed to predict the prognosis of liver disease [14, 15].

The indocyanine green (ICG) clearance test

ICG is a tricarbocyanine dye that binds primarily to albumin and distributes uniformly in the blood within a few minutes after injection. ICG is exclusively cleared by hepatocytes and excreted into the bile without biotransformation by multidrug-resistance-associated protein 2 [2]. The hepatic blood flow directly affects the ICG uptake by hepatocytes, and the clearance of ICG from the blood stream is similar to that of bilirubin, hormones, drugs and toxins. Therefore, it reflects several liver functions, including the blood flow-dependent clearance and transporter functions [4, 16, 17].

Under normal physiological conditions, the $ICGR_{15}$ is a reliable single parameter that can be used for the evaluation of liver function. However, several potential factors may affect the results, such as the presence of an arterial shunt, thrombosis and excessive bilirubin levels. Furthermore, the technical procedure for $ICGR_{15}$ measurement involves blood withdrawal 15 min after injection, and inaccurate timing can influence the results. Of late, pulse-dye densitometric measurement for plasma ICG evaluation has become available for clinical use [18]. This is a very simple method that automatically calculates the $ICGR_{15}$ after a single injection of ICG (Fig. 1). As such, it may provide a more accurate assessment of the liver function compared with the classical blood sampling methods.

The monoethylglycinexylidide (MEGX) test

The MEGX test is a dynamic liver function test that evaluates the hepatic conversion of lidocaine to MEGX [17, 19, 20]. Lidocaine is metabolized primarily by the

Table 3 Formula for calculating the model for end-stage liver disease (MELD) score and other modified scores

MELD = 9.57 × loge(creatinine, mg/dl) + 3.78 × loge (total bilirubin, mg/dl) + 11.2 × loge (INR) + 6.43
MELDNa = MELD + 1.59 × X (135 − serum sodium)
iMELD = MELD score + (age × 0.3) − (0.7 × serum sodium) + 100
MESO index = [MELD/Na (mEq/l)] × 100
UKELD = 5 × [1.5 × loge (INR) + 0.3 × loge(creatinine, μmol/l) + 0.6 × loge (bilirubin, μmol/l) − 13 × loge(serum sodium, mmol/l) + 70]
Re-weighted MELD = 1.266 loge (1 + creatinine, mg/dl) + 0.939 loge (1 + bilirubin, mg/dl) + 1.658 loge (1 + INR)

MELDNa model for end-stage liver disease with the incorporation of serum sodium, *iMELD* integrated model for end-stage liver disease, *MESO* model for end-stage liver disease to sodium, *UKELD* United Kingdom end-stage liver disease, *INR* international ratio

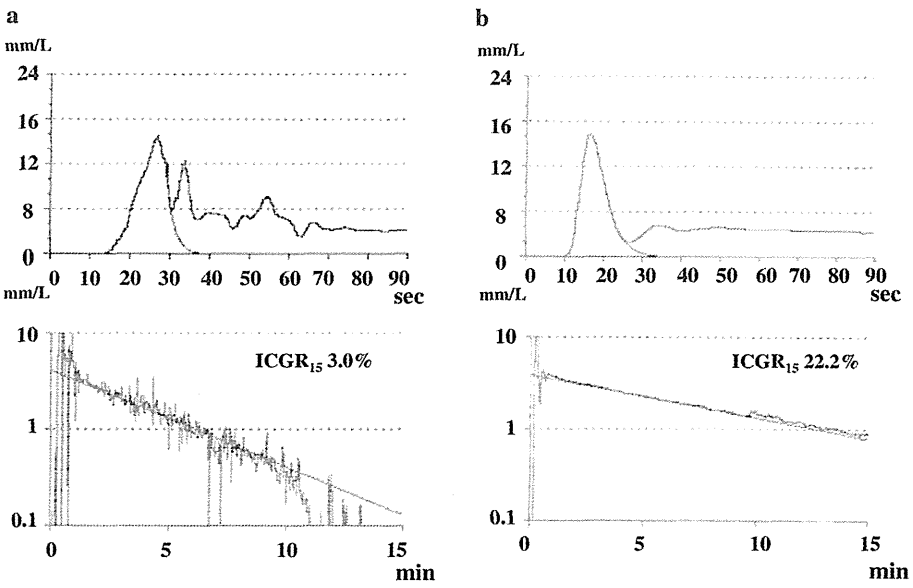

Fig. 1 Measurement of the indocyanine green retention rate at 15 min ($ICGR_{15}$) using pulse-dye densitometry (DDG-3300K; Nihon Koden, Tokyo, Japan). Representative examples of normal (**a**) and poor liver function (**b**). The *vertical axis* of *each graph* represents the plasma ICG concentration

liver cytochrome P450 system through sequential oxidative N-dealkylation, with MEGX being the major initial metabolite in humans. The MEGX test is a real-time measurement of hepatic metabolic function, and is used in the field of transplantation and critical care medicine, as well as in various experimental models. However, the MEGX test has wide interindividual variability. Patients require constant monitoring, and the test is not suitable for patients in the initial stages of chronic hepatitis [19].

The galactose elimination capacity test

The galactose elimination capacity test is used to determine the metabolic capacity of the liver [2, 21]. This test represents the liver function and adequately reflects the clinical prognosis [21]. However, it involves the time-consuming process of taking repeated timed blood samples, making it undesirable for routine clinical use. In addition, the galactose kinetics are affected by anaerobic respiration and can give false-positive results.

The ^{13}C liver-function breath tests

The ^{13}C liver-function breath tests have been investigated as noninvasive tools for the evaluation of liver function in patients with acute and chronic liver diseases (Table 4). However, these tests are not widely used in clinical practice for several reasons [22]. An intra-individual variability of 8–15 % was reported for each ^{13}C substrate, and this can directly affect the assessment [22]. Furthermore,

Table 4 ^{13}C-Liver-function breath tests

Cytosolic ^{13}C-liver-function breath tests	
^{13}C-phenylalanine	(^{13}C-PBT)
^{13}C-galactose	(^{13}C-GBT)
Mitochondrial ^{13}C-liver-function breath tests	
^{13}C-alpha-ketoisocaproate	(^{13}C-KICA)
^{13}C-methionine	(^{13}C-MeBT)
^{13}C-octanoate	(^{13}C-OBT)
Microsomal ^{13}C-liver-function breath tests	
^{13}C-aminopyrine	(^{13}C-ABT)
^{13}C-methacetin	(^{13}C-MBT)

physiological activities, such as movement and food intake, and interindividual variability, also affect the clinical assessment. Many other physiological, anatomical and biochemical factors may also affect the recovery of the label in the breath. Although these tests have been widely investigated, unstable measurements hamper their widespread clinical use.

Other parameters for evaluating protein production (Table 5)

Rapid turnover protein levels [prealbumin and retinol binding protein (RBP)]

Prealbumin is a 62-kDa homotetrameric protein, also known as transthyretin. It is produced not only by the liver, but also by other tissues, such as the choroid plexus and

Table 5 Other parameters for the assessment for protein production

Category	Parameters
Rapid turnover protein	Prealbumin
	Retinol binding protein
Lipid protein	Apolipoprotein A
	Apolipoprotein B
Anticoagulant	Antithrombin III
Amino acid balance	Fischer ratio
	BTR

BTR branched-chain amino acids to tyrosine ratio

retinal pigment epithelium. The prealbumin level may reflect the nutritional status, because its protein half-life, which is 1–3 days, is extremely short compared to that of albumin or transferrin [23]. It is believed to more closely reflect the recent dietary intake rather than the overall nutritional status. In addition, prealbumin can represent the recent liver protein production within the past few days and predict the risk of postoperative surgical complications [24].

RBP is a 21-kDa, single polypeptide chain that circulates as a 1:1 molar complex with prealbumin [25]. RBP gene expression has been observed not only in the liver, but also in epididymal fat and other organs, such as the kidneys, lungs, etc. [26]. The half-life of plasma RBP is also very short, at 12 h. Because of their short half-lives, both the prealbumin and RBP levels can reflect the status of protein production by the liver. If the plasma levels of both proteins do not improve after the provision of nutritional support, it can be concluded in real-time that there is liver dysfunction.

Apolipoproteins

Apolipoproteins are also produced chiefly by the liver. They decrease with an increase in disease severity in patients with cirrhosis [27, 28]. The severity of liver disease plays an important role in maintaining the serum lipid levels. Although the apolipoprotein levels represent the recovery of residual liver function after hepatectomy, the plasma levels do not precisely correlate with volume recovery [29, 30]. Therefore, the clinical value of apolipoprotein levels as a parameter for assessing the liver function is limited to a resting stable status, such as that during preoperative evaluation.

Antithrombin III (AT III)

AT III is an anticoagulant that neutralizes thrombin and other activated serine proteinases comprising the coagulation pathway [31]. The plasma ATIII levels correlate with the liver function [32, 33]. ATIII also correlates with the $ICGR_{15}$ in patients with hepatocellular carcinoma (HCC) who undergo liver resection [34]. In fact, an interesting formula has been reported, wherein the sum of the ATIII levels and $ICGR_{15}$ tends to be 100 %. In fact, AT III is a predictive factor for the prognosis of HCC patients [35].

The branched-chain amino acids (BCAA) to tyrosine ratio (BTR)

The BTR is an alternative indicator for the Fischer ratio, which is the ratio of BCAA to aromatic amino acids (AAA) [36, 37]. Because the measurement of AAA is too complex to use routinely, the BTR is preferred for clinical use [36]. The balance between the BTR and albumin plays an important role not only as a risk factor for surgical complications, but also as a predictive factor for the prognosis in HCC patients [24]. It may be useful to identify specific patients who would benefit from receiving supplemental BCAA to increase their postoperative survival [8].

Indirect parameters for evaluating liver fibrosis and cirrhosis

Diagnostic tests for evaluating liver fibrosis and cirrhosis can potentially represent the liver function [38, 39]. The liver function deteriorates along with the inflammatory process, and the two parameters correlate well with each other. Therefore, any parameters or indicators of liver fibrosis and cirrhosis can reflect the liver function. Below, we present a review of the representative parameters for liver fibrosis.

The aspartate aminotransferase-to-platelet ratio index (APRI)

The APRI is reportedly an indirect biochemical marker of hepatic fibrosis. It is based on routine laboratory parameters and reflects alterations in hepatic function [40]. The APRI is calculated by the following formula: aspartate aminotransferase levels/platelet counts (10^9/L) × 100. A recent meta-analysis suggested that the APRI can identify hepatitis C-related fibrosis with a moderate degree of accuracy [41].

Hyaluronic acid (HA)

Plasma HA is a glycosaminoglycan that is eliminated from plasma via binding to a specific endothelial receptor [42, 43]. The HA level reflects sinusoidal endothelial cell function [44, 45], which can be damaged in patients with portal hypertension. In addition, it can function as a marker

for the severity of liver disease [46] and as a predictive factor for liver dysfunction after hepatectomy [47]. The HA level also correlates with multiple liver function indicators [47]. However, it cannot be used for evaluations during an acute phase, such as that immediately after hepatectomy.

Collagens

Of the several procollagen and collagen fragments proposed as markers, only the N-terminal propeptide of type III procollagen (PIIINP) has achieved limited clinical application [38, 43]. However, it should be emphasized that PIIINP is not a liver-specific biomarker. Furthermore, a study failed to prove the utility of PIIINP as an independent marker for liver fibrosis; therefore, the clinical application of PIIINP has not received widespread acceptance [38]. Type IV collagen is found predominantly in the basement membrane of the liver, and various studies have shown its diagnostic value for the monitoring of liver fibrosis [43]. However, the diagnostic advantage of type IV collagen over other fibrotic biomarkers has not been proven. Theoretically, the type IV collagen levels reflect the extent of fibrosis in patients with alcoholic liver disease, because the fibrosis in such patients is mostly seen in the perivenular and pericellular regions.

Imaging modalities for the assessment of liver function

^{99m}Tc-galactosyl serum albumin scintigraphy

^{99m}Tc-diethylenetriamine-pentaacetic acid-galactosyl human serum albumin (GSA) binds to asialoglycoprotein receptors that are exclusively expressed on the hepatocyte sinusoidal surface facing the space of Disse [2, 3, 17]. The liver is the only uptake site for ^{99m}Tc-GSA, making ^{99m}Tc-GSA an ideal agent for use in receptor-targeted functional liver scintigraphy [48, 49]. While many different parameters can be calculated from different kinetic models, some are too complex to use routinely. However, the hepatic uptake ratio of ^{99m}Tc-GSA and the blood clearance ratio are commonly used parameters in planar dynamic ^{99m}Tc-GSA scintigraphy. We recently developed a simple software program to automatically calculate the pixel counts of the area between the hepatic curve and heart curve from 3 to 15 min (Fig. 2a) [50]. In patients with predominant cirrhosis, a functional volume evaluation of ^{99m}Tc-GSA counts using single photon emission computed tomography has proven more accurate in predicting the remnant liver function compared with classical computed tomography volumetry [51]. However, the liver function can be distributed heterogeneously in tumor-bearing livers (Fig. 2b–d), and the total functional liver volume does not necessarily correlate with intrinsic liver function. In addition, the test is not suitable for patients with hepatocellular failure secondary to biliary obstruction [2].

^{99m}Tc-Mebrofenin hepatobiliary scintigraphy

^{99m}Tc-mebrofenin circulates in an albumin-bound form and dissociates from albumin after uptake into hepatocytes through organic anion transporters [2]. ^{99m}Tc-mebrofenin undergoes biliary excretion without undergoing biotransformation, similar to ICG. Previous clinical studies compared the effectiveness of the ICG clearance test with that of ^{99m}Tc-mebrofenin hepatobiliary scintigraphy in patients undergoing liver resection and showed a good correlation between the two [52]. Furthermore, ^{99m}Tc-mebrofenin hepatobiliary scintigraphy has been validated as a tool for measuring the total liver function and functional remnant liver before liver surgery [52].

Transient elastography (TE)

Liver fibrosis can be estimated using ultrasound TE, which measures the velocity of a low-frequency (50 Hz) elastic shear waves penetrating the liver [39, 53]. Several advantages of TE have been reported, such as low invasiveness, fast acquisition of results, and portability that enables testing at the bedside and in outpatient departments [54]. However, unreliable and unrepeatable measurements caused by host obesity, anatomical difficulties such as a narrow intercostal space, and inadequate operator experience have also been reported [54]. Further clinical studies are required to validate this imaging method to determine whether it can be a suitable alternative to blood tests.

Indications for liver resection and surgical procedures in HCC patients

The indications for liver resection and surgical procedures in HCC patients can be determined using the Makuuchi criteria (Fig. 3), which are based on the existence of uncontrolled ascites, the serum bilirubin levels and the $ICGR_{15}$ [16, 55]. Briefly, if the serum bilirubin levels are normal, the criteria permit right hepatectomy or trisectorectomy when the $ICGR_{15}$ is <10 %, left hepatectomy or sectoriectomy when the $ICGR_{15}$ is <20 %, subsegmentectomy or Couinaud's segmentectomy when the $ICGR_{15}$ is <30 %, limited resection when $ICGR_{15}$ is <40 % and enucleation when the $ICGR_{15}$ is ≥ 40 %. Multidetector computed tomography has recently been used to calculate the precise liver volume. Volumetric evaluation has been recommended before major hepatic resections involving more than four segments [56]. The total liver volume is estimated on the basis of the body surface area of the

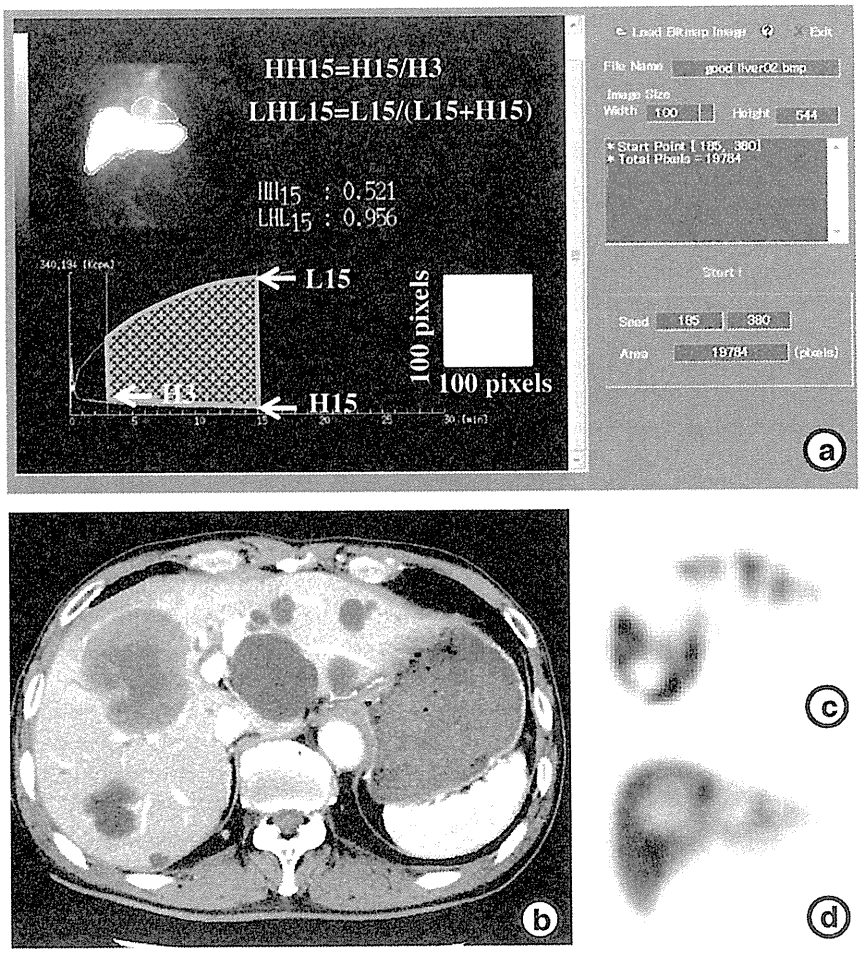

Fig. 2 An ^{99m}Tc-diethylenetriamine-pentaacetic acid-galactosyl human serum albumin (GSA) image after applying an auto-calculating software program for pixel counts of the area between the hepatic curve and heart curve (ABC) from three to 15 min (**a**). A representative case of axial computed tomography (CT) (**b**) and GSA single photon emission computed tomography (SPECT) (**c**, **d**) showing multiple parenchymal loss (**b**) and heterogenous GSA accumulation (**c**, **d**). A GSA SPECT axial image (**c**) and a coronal image (**d**) of the same patient

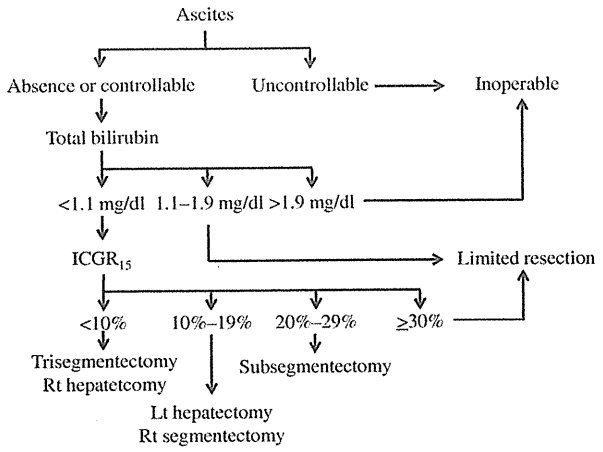

Fig. 3 An algorithm of the surgical indications for hepatocellular carcinoma according to the Makuuchi criteria

patients using the following formula: liver volume (cm^3) = 706 × body surface area (m^2) + 2.4 [57]. According to an expert consensus statement in the Vauthey report [56], the minimum liver volume required after liver resection was estimated to be approximately 20 % for a normal liver, 30 % for an injured liver and 40 % for a liver with well-compensated hepatic fibrosis and cirrhosis (Table 6). With regard to a safe remnant liver volume, Poon and Fan proposed that a CT volumetric evaluation should be considered in patients who show an $ICGR_{15}$ of 14–20 % [58]. These patients are considered to have a borderline liver function reserve for major hepatectomy. Unfortunately, they did not define the exact liver volume required for a safe major hepatectomy, and the safe remnant liver volume is considered to be approximately 30–40 % (Fig. 4).

In the Barcelona clinic liver cancer (BCLC) algorithm for HCC treatment, surgical treatment is limited to patients with good liver function and with a single tumor <5 cm in diameter [59]. In addition, surgical candidates for liver resection should not have portal hypertension, which is defined by the presence of a hepatic venous pressure gradient of >10 mmHg, esophageal varices or splenomegaly with a platelet count of <100,000/mm^3 (Fig. 5). Although

Table 6 Minimum residual liver volume for liver resection as proposed by a consensus statement

Estimated total liver volume (cm^3) = 706 × body surface area (m^2) + 2.4	
Liver condition	Minimum residual liver volume (%)
Normal	20
Injured liver	30
Well-compensated hepatic fibrosis and cirrhosis	40

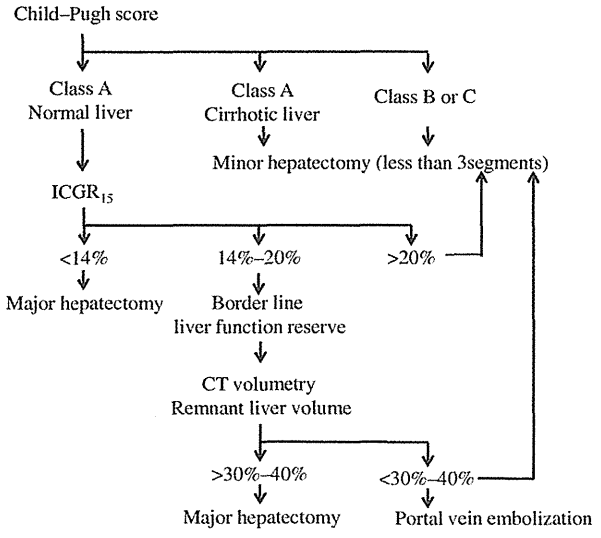

Fig. 4 An algorithm of the surgical procedure for hepatocellular carcinoma as proposed by Poon and Fan

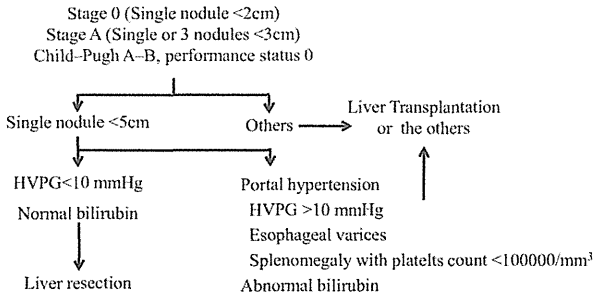

Fig. 5 A modified algorithm of the surgical procedure for hepatocellular carcinoma according to the Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy. *HVPG* hepatic venous pressure gradient

the oncological benefit of anatomical HCC resection has been reported [60, 61], the survival benefit of resection in patients with accompanying cirrhosis is debatable [62]. A surgical plan for HCC patients should take into consideration both the oncological behavior and liver function.

Summary

We herein reviewed the relevant literature concerning the parameters and tests currently used for liver function assessment. No single parameter or test can represent liver function entirely because of the wide variety of liver functions. A systematic combination of each parameter and test is necessary to provide a meticulous evaluation. The integration of available diagnostic tests and establishment of a classification system is required to thoroughly evaluate the liver function and ensure the safety of surgical procedures.

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Conflict of interest The authors declare that they have no conflict of interest to disclose.

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ORIGINAL ARTICLE

An immunohistochemical marker panel including claudin-18, maspin, and p53 improves diagnostic accuracy of bile duct neoplasms in surgical and presurgical biopsy specimens

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Abstract Biliary tract cancers have an extremely poor outcome, and specific diagnostic markers and effective treatments are needed urgently. In this study, we assessed the capacity of panel of immunohistochemical markers including claudin-18, maspin, and p53 to distinguish biliary tract carcinoma and biliary intraepithelial neoplasia (BilIN) from non-neoplastic epithelium. We performed a retrospective study of 66 biliary tract cancer specimens and 63 specimens with non-neoplastic lesions. Of the surgical specimens, 96.7 % with adenocarcinoma/BilIN were detected as neoplastic, and all 63 specimens histologically diagnosed as non-neoplastic lesion were detected as non-neoplastic with high sensitivity (91.1 %) and specificity (100 %). Of presurgical endobiliary forceps biopsy specimens, all with adenocarcinoma/BilIN and only 1 of the 19 with a non-neoplastic lesion were distinguished as neoplastic with high sensitivity (100 %) and specificity (94.7 %). Moreover, this panel provided good separation of neoplasm from malignancy-undetermined atypical epithelium (18/21, 85.7 %). This panel achieves a more reliable distinction of biliary tract cancers and BilINs from non-neoplastic epithelia in both surgical and biopsy specimens than immunohistochemical analysis with single antibodies and is useful in supporting a diagnosis of adenocarcinoma and BilIN.

Keywords Claudin-18 · Maspin · p53 · Human bile duct cancer · Early diagnosis

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Introduction

The incidence of biliary tract cancers, comprising gallbladder, bile duct, and ampullary cancer, has been increasing worldwide over the past several decades and in the USA over the last decade [1, 2]. In Japan, the morbidity associated with these cancers has also increased, and more than 18,000 people died of this cancer in 2012 [3].

Generally, the overall prognosis for biliary tract cancer is poor. Although currently only surgical resection may be curative, the curative resection rate has remained low at approximately 40 % [4]. Therefore, early detection and preoperative confirmation of the malignant diagnosis is vitally important in improving prognosis [5]. The gold standard method of diagnosis requires endobiliary forceps biopsy and percutaneous liver biopsy. However, histopathological examination of biopsy tissues in clinical practice can be challenging because of a limited amount of material, crush artifacts, and the presence of confounding acute and chronic inflammatory epithelial changes [6, 7].

The need for accurate diagnostic methods has led to the exploration of immunohistochemical markers to distinguish

between benign atypia and malignancy [8–13]. In the present study, we examined the potential of three molecules, claudin-18 (cldn18), maspin, and p53, to serve as immunohistological diagnostic markers for bile duct cancers, biliary intraepithelial neoplasia (BilIN), and ampullary cancer, which can be difficult to diagnose by histology alone. BilIN is a flat-type premalignant or in situ neoplastic lesion of the biliary tract that was first documented in 2005 and has been recently included in the WHO classification of 2010 as intraductal papillary neoplasm of the bile duct (IPNB) [14–16]. BilIN occurs in intrahepatic and extrahepatic bile ducts and sometimes result from disorders of the biliary tract, such as hepatolithiasis, choledochal cysts, and primary sclerosing cholangitis. Biliary tract cancers progress through multistep carcinogenesis, with multiple molecular events such as KRAS and GNAS mutation and p53 overexpression, and BilIN and IPN are precursor lesions [15, 17]. BilIN is subdivided into BilIN-1, BilIN-2, and BilIN-3 according to the degree of cellular atypia and architectural disturbance. BilIN-1 and BilIN-2 correspond to low and intermediate grades, respectively. BilIN-3 is high grade and equivalent to carcinoma in situ.

Claudins are tight junction resident transmembrane proteins that are present in epithelial and endothelial cells and in derived neoplastic cells [18]. Aberrant expression of a number of claudins has been reported in various carcinomas [19, 20]. Cldn18 is detected in gastrointestinal and lung tissues [21–23]. In pancreatic ductal adenocarcinoma, cldn18 is overexpressed and has been identified as a potential diagnostic marker [24]. In the biliary tract, multivariable analysis demonstrated that positive cldn18 expression is an independent risk factor for lymph node metastasis [25]. Recently, we reported that cldn18 is primarily regulated at the transcriptional level via specific protein kinase C signaling pathways and that its expression is modified by DNA methylation [26].

Mammary serine protease inhibitor, otherwise known as maspin, is a member of the serine protease inhibitor superfamily and was identified as a tumor suppressor in mammary tissue in 1994 [27]. However, subsequent studies have revealed its tumor-suppressive properties to be complex and dependent on factors such as genetic background, type of cancer, and the expression of maspin (or lack thereof) in the corresponding normal tissue. Interestingly, both methylation and demethylation of the *maspin* promoter have been reported to influence its expression [28]. Some studies demonstrated an association between hypermethylation of the *maspin* promoter and loss of maspin expression in colonic and ovarian cancers [29, 30]. Others reported that demethylation was associated with maspin overexpression in gastric cancer [31]. In biliary tract cancer, demethylation of the *maspin* promoter and aberrant maspin expression has been reported [32]. In pancreatic ductal adenocarcinoma, overexpression of maspin is associated with lower postoperative survival [33]. In the gallbladder, use of an immunohistochemical panel including maspin has been reported to distinguish adenocarcinoma from benign/reactive epithelium [34].

Mutation of the *p53* gene is a key event in the carcinogenesis of many different types of tumors. The presence of this genetic abnormality in biliary tract cancer has been suggested in various investigations that used immunohistochemical and molecular epidemiological methods [35, 36].

Currently, accurate cancer detection including localization is needed to improve the prognosis of patients with bile duct cancers. In this study, we used immunohistochemical methods to document the expression of cldn18, maspin, and p53 in bile duct carcinomas, BilINs, and ampullary carcinoma in surgical specimens and analyzed the diagnostic utility of this immunohistochemical panel in presurgical bile duct biopsy specimens.

Materials and methods

Surgical specimens

A total of 66 biliary tract cancer specimens obtained by surgical excision from 1999 to 2011 were retrieved from the pathology file of Sapporo Medical University Hospital, Sapporo, Japan. Their clinicopathological characteristics are summarized in Table 1. Gallbladder cancer was excluded from the present study because presurgical biopsies had not been performed. The histological type of all cancers was adenocarcinoma. The cancer staging system was based on both the UICC classification (7th edition) and the Japanese Society of Biliary Surgery classification (fifth edition). Among the 66 adenocarcinomas, 25 (9 intrahepatic, 5 hilar extrahepatic bile duct, 7 distal extrahepatic bile duct, and 4 ampulla of Vater) had flat intraepithelial neoplastic lesions around invasive carcinomas. These intraepithelial lesions consisted of precursor lesions, that is, true BilIN, and superficial spreading lesions that were difficult to differentiate. Thus, in the present study, all intraepithelial components were classified as BilIN-1, BilIN-2, and BilIN-3 according to the degree of cell atypia. In addition, 63 specimens with non-neoplastic epithelia from cases of adenocarcinoma (25 intrahepatic, 10 hilar extrahepatic bile duct, 21 distal extrahepatic bile duct, and 7 ampulla of Vater) were selected as a control group. All slides were independently evaluated by three pathologists (KY, TA, and MM). Discordant cases were discussed, and a consensus was reached.

Immunohistochemical staining of surgical specimens

The hematoxylin and eosin (H&E)-stained slides from all cases were reviewed to select representative sections. New sections from paraffin blocks were examined by the labeled polymer method. Sections were deparaffinized, rehydrated, moistened with phosphate-buffered saline (PBS; pH 7.4),

Table 1 Clinicopathological features of biliary tract cancers

Total (N=66)		Intrahepatic bile duct carcinoma (N=27)		Extrahepatic bile duct carcinoma (N=32)		Ampullary carcinoma (N=7)	
Age (range, median)	39–84, 68.5	T grade (UICC)		T grade (UICC)		T grade (UICC)	
Sex		T1/T2	22	Tis/T1/T2	18	Tis/T1/T2	4
Male	47	T3/T4	5	T3/T4	14	T3/T4	3
Female	19	T grade[b]		T grade[b]		T grade[b]	
Location		T1/T2	14	T1/T2	11	T1/T2	2
Intrahepatic	27	T3/T4	13	T3/T4	21	T3/T4	5
Extrahepatic	32	Lymph node metastasis		Lymph node metastasis		Lymph node metastasis	
Hilar	11	Negative	24	Negative	20	Negative	4
Distal	21	Positive	3	Positive	12	Positive	3
Ampulla of Vater	7	Stage group (UICC)		Stage group (UICC)		Stage group (UICC)	
Tumor size		I/II	20	0/IA/IB/II	15	0/IA/IB/II	2
≦3 cm	29	III/IV	7	IIA/IIB/IIIA/IIIB	17	IIA/IIB/IIIA/IIIB	5
>3 cm	25	Stage group[b]		Stage group[b]		Stage group[b]	
Unknown	12	I/II	13	I/II/III	17	I/II/III	3
Histological type		III/IVA/IVB	14	IVA/IVB	15	IVA/IVB	4
Well	33	Lymphatic invasion		Lymphatic invasion		Lymphatic invasion	
Moderately	16	Negative	–	Negative	13	Negative	2
Poorly	17	Positive	–	Positive	19	Positive	5
BilIN[a]		Venous invasion		Venous invasion		Venous invasion	
BilIN-1	8	Negative	18	Negative	16	Negative	3
BilIN-2	11	Positive	9	Positive	23	Positive	4
BilIN-3	6			Interstitial connective tissue		Interstitial connective tissue	
				Medullary	4	Medullary	1
				Intermediate	20	Intermediate	6
				Scirrhous	8	Scirrhous	0

[a] Flat intraepithelial neoplastic lesion around invasive carcinoma classified as BilIN-1, BilIN-2, and BilIN-3 according to the degree of cell atypia

[b] General rules for surgical and pathological studies on cancer of the biliary tract (fifth edition) by the Japanese Society of Biliary Surgery

and then pretreated in an autoclave at 121 °C for 5 min in 10 mM citrate buffer (pH 6.0), followed by 30 min incubation with antibodies to the following antigens in an automated immunostaining system (Dako Autostainer; Dako, Carpinteria, CA, USA): cldn18 (Invitrogen, Carlsbad, CA; polyclonal, ×100), maspin (BD, Franklin Lakes, NJ; G167-70, ×50), and p53 (Dako, DO-7, ×50). Maspin immunoreactivity was independently evaluated in the cytoplasm (C) or nucleus (N). The intensity of staining was assessed as strong (3), moderate (2), weak (1), or negative (0). The proportion of neoplastic cells stained was recorded as 0 (no staining), 1 (1–10 %), 2 (11–20 %), 3 (21–30 %), 4 (31–40 %), 5 (41–50 %), 6 (51–60 %), 7 (61–70 %), 8 (71–80 %), 9 (81–90 %), or 10 (91–100 %). Because neoplasm heterogeneity caused variable immunoreactivity in each case, we established a multiplication score for improvement of accuracy: The minimum score was intensity 0×proportion 0 (multiplication score 0), and the maximum was intensity 3×proportion 10 (multiplication score 30). Several representative fields were examined.

Double-staining immunohistochemistry

For double immunostaining, paraffin-embedded tissue sections were deparaffinized in xylene (10 min, two times) and rehydrated through a graded ethanol series. Antigen retrieval was performed by immersing sections in 10 mM Tris-1 mM EDTA buffer (pH 9.0) and boiling in a microwave oven (95 °C, 30 min). After washing of the sections with PBS (5 min, three times), they were allowed to cool at room temperature. They were then incubated in 3 % hydrogen peroxide for 10 min to inactivate endogenous peroxidase. After washing in PBS (5 min, three times), they were incubated with anti-maspin antibody (BD, G167-70, ×50) overnight at 4 °C. The following day, the sections were washed in PBS (5 min, three times), and immunostaining was performed by a standard immunoperoxidase technique (Histofine SAB-PO Kit, Nichirei Co., Tokyo, Japan) with a BCIP/NBT substrate system (Dako Laboratories) as chromogen, according to the manufacturer's instructions. After the sections were washed in distilled water (5 min, three times), antigen retrieval

was performed by immersing the sections in 10 mM Tris-1 mM EDTA buffer (pH 9.0) and boiling in a microwave (95 °C, 10 min). The sections were washed with PBS (5 min, three times) and allowed to cool to room temperature. Subsequently, the sections were incubated with anti-cldn18 antibody (Invitrogen, polyclonal, ×100) overnight at 4 °C. The following day, after the sections were washed in PBS (5 min, three times), immunostaining was performed with the Dako REAL™ EnVision™ Detection System (Dako ChemMate, Glostrup, Denmark) with diaminobenzidine (Dako Laboratories) as the chromogen, according to the manufacturer's instructions. Sections were then counterstained with hematoxylin, dehydrated, and mounted.

Immunohistochemical analysis of presurgical biopsy specimens

As an additional study, immunohistochemical analysis was performed on 58 samples (18 adenocarcinomas, 21 malignancy-undetermined atypical epithelia, and 19 non-neoplastic lesions) taken from presurgical extrahepatic bile duct forceps biopsies and 7 samples (4 adenocarcinomas and 3 non-neoplastic lesions) taken from presurgical percutaneous biopsies. All specimens of malignancy-undetermined atypical epithelium showed nuclear atypia and turned out to be adenocarcinoma by histological examination of the subsequent surgical specimens. The 19 specimens with non-neoplastic lesions from endobiliary forceps biopsies comprised 5 specimens of IgG4-related sclerosing cholangitis, 5 of primary sclerosing cholangitis, and 9 of nonspecific fibrosis/inflammation. All three specimens of non-neoplastic lesions from percutaneous liver biopsies concerned nonspecific fibrosis/inflammation. None of the patients had a stent when the biopsy was performed. The immunohistochemical protocol was the same as that described above. Because of the small amount of epithelium in biopsy specimens, any immunoreactivity in epithelial cells was regarded as positive regardless of the multiplication score. A case with one or more positive atypical epithelia was given a binary value of 1, while absence of positive atypical epithelia was given a binary value of 0.

Statistics

A three-step analysis was used for the surgical specimens. In the first step, cutoff values were calculated for the multiplication scores of cldn18, maspin (N), and p53 that would distinguish the following: (i) adenocarcinoma from non-neoplastic epithelium, (ii) BilIN from non-neoplastic epithelium, and (iii) neoplasm (adenocarcinoma/BilIN) from non-neoplastic epithelium. In the second step, other cutoff values were calculated for the combined multiplication scores from cldn18, maspin (N), and p53 that would distinguish neoplastic (adenocarcinoma/BilIN) from non-neoplastic epithelium. Third, for every antibody, the multiplication score was converted to its respective binary value using cutoff values obtained in the first step as the threshold. The score with the highest sensitivity and specificity was used to define the receiver operator characteristic (ROC) curve, and the area under the receiver operator characteristic curve (AUC) was calculated. We used 95 % confidence intervals (CIs) to test the hypothesis that AUC is 0.5. For presurgical biopsy specimens, ROC curve analysis was performed to calculate the best binary value in the combination of cldn18, maspin (N), and p53. All statistical analyses were performed with SPSS statistics ver. 20.

Results

Patient characteristics

The study population of 66 patients with biliary tract cancers consisted of 47 men and 19 women, ranging at the time of diagnosis between 39 and 84 years of age (Table 1). The median age of the patients was 68.5 years. The number of patients according to UICC stage was as follows: intrahepatic bile duct cancer I/II $n=20$ and III/IV $n=7$, and extrahepatic bile duct and ampullary carcinoma 0/IA/IB/II $n=17$ and IIA/IIB/III/IV $n=22$. Cases of BilIN were classified as 8 of BilIN-1, 11 of BilIN-2, and 6 of BilIN-3 as described in the "Materials and methods." None of the patients had papillary lesions identified as IPNB.

Cldn18 expression in surgical specimens

First, we examined the immunochemistry of the surgical specimens for cldn18, maspin, and p53 independently. In the biliary tract tissues, immunostaining of cldn18 was observed in the basolateral membrane of the neoplastic cells (Fig. 1). In contrast, staining for cldn18 was almost absent in non-neoplastic epithelial cells. To maximize reproducibility and accuracy of the immunohistochemical evaluation, we defined a parameter, designated as the multiplication score, which was calculated by multiplying intensity (4 grades) and proportion (11 grades) of immunoreactivity. The multiplication scores for cldn18 in adenocarcinoma, BilIN-3, BilIN-2, BilIN-1, and non-neoplastic epithelium were (mean±SD/median) 22±6.8/24, 26±2.8/27, 25±6.1/27, 23±9.1/27, and 0.97±2.3/0, respectively (Table 2).

In adenocarcinomas, the multiplication score was lower because differentiation of the neoplasm was poor. Multiplication scores in well, moderately, and poorly differentiated adenocarcinoma were (mean±SD/median) 23±5.8/24, 21±8.2/24, and 17±7.5/18, respectively (Table 3

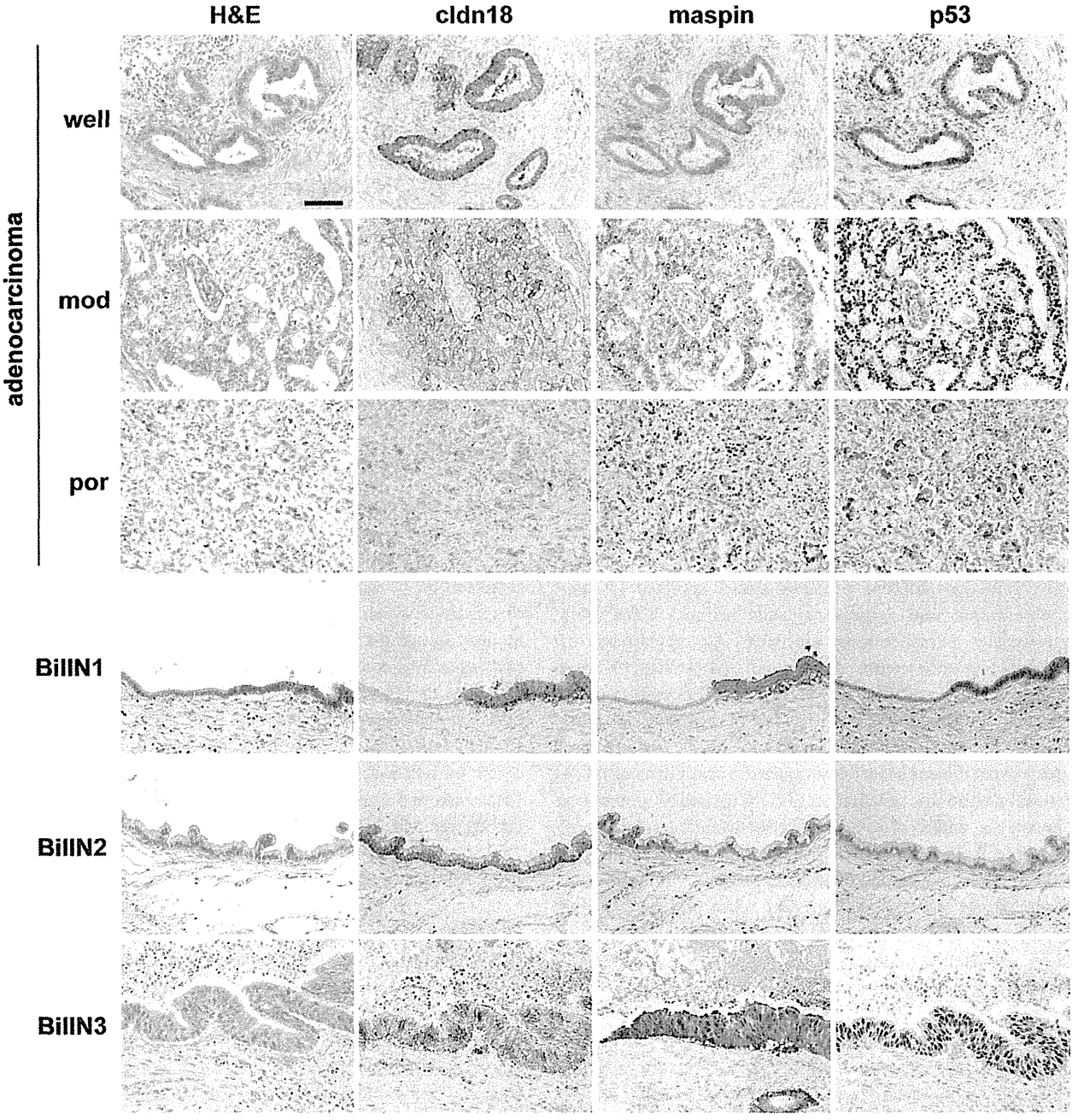

Fig. 1 H&E staining and immunohistochemical staining in surgical specimens of well, moderately (mod), and poorly (por) differentiated bile duct adenocarcinoma and BilIN-1, BilIN-2, and BilIN-3. Cldn18 was expressed on basolateral membranes of the epithelial cells in adenocarcinoma and BilIN-1, BilIN-2, and BilIN-3. Maspin was also expressed in both the cytoplasm and nucleus of the epithelial cells in adenocarcinoma and BilIN-1, BilIN-2, and BilIN-3. p53 was expressed in the nucleus of the epithelial cells in some specimens of adenocarcinoma and BilIN-2 and BilIN-3. In the non-neoplastic epithelial cells adjacent to BilIN, none of the three antibodies caused staining

and Supplementary Fig. S1). Cldn18 was distributed along the entire cell membrane of most cells in well-differentiated adenocarcinomas and expressed at least in part on the cell surfaces of poorly differentiated adenocarcinoma cells (Fig. 1). There were no significant changes in the cldn18 expression patterns in BilIN-1, BilIN-2, and BilIN-3.

Maspin expression in surgical specimens

Immunostaining of maspin was observed in both the cytoplasm and nucleus of the neoplastic cells (Fig. 1). Apart from bile duct epithelium, some non-neoplastic hepatic cells and duodenal epithelial cells were positive

Table 2 Results of the immunohistochemical evaluation of cldn18, maspin, and p53 in surgical specimens

			Intensity		Proportion		Multiplication score	
Antibody	Histological type	Number	Mean±SD	Median	Mean±SD	Median	Mean±SD	Median
cldn18	Adenocarcinoma	66	2.8±0.5	3	7.7±1.9	8	22±6.8	24
	BilIN-3	6	3±0	3	8.7±1.0	9	26±2.8	27
	BilIN-2	11	3±0	3	8.3±2.0	9	25±6.1	27
	BilIN-1	8	2.6±0.70	3	8.1±2.1	9	23±9.1	27
	Non-ne	63	0.48±0.73	0	0.73±1.6	0	0.97±2.3	0
Maspin (C)	Adenocarcinoma	66	2.3±1.2	3	5.0±3.2	5.5	14±9.7	15
	BilIN-3	6	2.7±0.5	3	6.2±2.9	6	17±9.5	15
	BilIN-2	11	2.3±1.1	3	5.0±3.7	5	15±11	15
	BilIN-1	8	1.6±1.3	2	2.6±2.8	2	7.3±8.6	4
	Non-ne	63	0.35±0.91	0	0.33±0.85	0	0.79±2.1	0
Maspin (N)	Adenocarcinoma	66	2.5±1.0	3	5.1±3.0	6	15. ± 9.4	18
	BilIN-3	6	3.0±0	3	5.7±1.6	6.5	17±4.8	19.5
	BilIN-2	11	2.0±1.3	3	4.5±3.6	5	13±11	15
	BilIN-1	8	1.9±1.3	2.5	3.8±3.0	3	10±9.6	6
	Non-ne	63	0.57±1.1	0	0.57±1.1	0	1.5±3.0	0
p53	Adenocarcinoma	66	1.7±1.3	2	2.7±3.1	1	7.4±9.5	3
	BilIN-3	6	1.2±1.1	1	2.2±2.4	1	4.3±6.3	1.5
	BilIN-2	11	0.64±1.1	0	0.73±1.7	0	2±5.1	0
	BilIN-1	8	0.63±1.1	0	1±2.3	0	2.9±6.9	0
	Non-ne	63	0	0	0	0	0	0

Non-ne non-neoplastic epithelium

for maspin in the nucleus. Multiplication scores for maspin (C) in adenocarcinoma, BilIN-3, BilIN-2, BilIN-1, and non-neoplastic epithelium were (mean±SD/median) 14±9.7/15, 17±9.5/15, 15±11/15, 7.3±8.6/4, and 0.79±2.1/0, respectively (Table 2). Multiplication scores for maspin (N) in adenocarcinoma, BilIN-3, BilIN-2, BilIN-1, and non-neoplastic epithelium were (mean±SD/median) 15±9.4/18, 17±4.8/19.5, 13±11/15, 10±9.6/6, and 1.5±3.0/6, respectively (Table 2). Multiplication scores for maspin (N) in well, moderately, and poorly differentiated adenocarcinoma were (mean±SD/median) 17±8.2/21, 12±10/10.5, and 14±9.9/12, respectively (Table 3 and Supplementary Fig. S1). There were no significant differences

Table 3 Results of the immunohistochemical evaluation of well, moderately, and poorly differentiated adenocarcinoma in surgical specimens

			Intensity		Proportion		Multiplication score	
Antibody	Differentiation	Number	Mean±SD	Median	Mean±SD	Median	Mean±SD	Median
cldn18	Well	33	2.9±0.4	3	8.0±1.5	8	23±5.8	24
	Moderately	16	2.7±0.7	3	7.8±2.2	8	21±8.2	24
	Poorly	17	2.2±0.7	2	7.2±2.1	8	17±7.5	18
Maspin (C)	Well	33	2.5±1.0	3	5.9±3.0	7	17±9.5	21
	Moderately	16	1.9±1.2	2.5	4.1±3.1	4	10±9.2	8.5
	Poorly	17	2±1.3	3	4±3.1	4	11±9.2	12
Maspin (N)	Well	33	2.6±0.8	3	5.8±2.6	7	17±8.2	21
	Moderately	16	2.1±1.3	3	4.1±3.4	3.5	12±10	10.5
	Poorly	17	2.4±1.0	3	4.8±3.2	5	14±9.9	12
p53	Well	33	1.8±1.3	2	2.9±3.2	1	7.8±9.6	3
	Moderately	16	1.6±1.2	2	2.9±3.2	1	7.5±9.3	2.5
	Poorly	17	1.6±1.3	2	2.1±2.8	1	5.8±8.7	2