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Feasibility of laparoscopic liver resection for caudate lobe: technical strategy and comparative analysis with anteroinferior and posterosuperior segments

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Abstract
Background Although laparoscopic liver resection (LLR) is now considered a standard procedure in peripheral segments, there are few reports on laparoscopic segment 1 (Sg1) resection. The aim of this study was to assess both safety and feasibility of Sg1 LLR.
Methods From 2000 to 2014, all patients who underwent LLR were identified from a prospective database. Patients with resection of Sg1 (Sg1 group) were compared with those with resection of anteroinferior segments (AI group: segments 3, 4b, 5, 6) or posterosuperior segments (PS group: segments 4a, 7, 8), in terms of tumor characteristics, surgical treatment, and short-term outcomes.
Results There were 15, 151, and 67 patients in Sg1, AI, and PS groups. Tumor size and tumor number were similar between the three groups ($p = 0.139$, $p = 0.102$). Operative time was significantly shorter in Sg1 (150 min) and AI group (135 min) compared with PS group (180 min) ($p = 0.021$). Median blood loss was notably higher in PS group (140 ml) compared with Sg1 group (75 ml) and AI group (10 ml) ($p = 0.001$). No mortality was observed in all groups. Postoperative complication rate was 20.0 % with Sg1 group, 14.6 % with AI group, and 20.9 % with PS group ($p = 0.060$). The rate of major complication was significantly higher in Sg1 group (13.3 %) and PS group (11.9 %) compared with AI group (4.0 %) ($p = 0.042$). Resection margins were clear in all Sg1 and PS group patients, whereas two (1.3 %) patients in AI group had R1 margins ($p = 0.586$).
Conclusion The laparoscopic approach of isolated resection located in the caudate lobe is a feasible and curative surgical option in selected patients.

Keywords Laparoscopic liver resection · Caudate lobe · Anteroinferior lesion · Posterosuperior lesion · Caudal approach

The caudate lobe (Couinaud's segment 1: Sg1) is a small liver segment consisting of three parts: Spiegel's lobe, paracaval portion, and caudate process. Sg1 is anatomically unique in that it is situated posteriorly in the liver and directly over the inferior vena cava (IVC), which makes this lobe not directly visible and less accessible for surgeons. In addition, Sg1 contains several thin hepatic veins draining directly into IVC, which increases the risk of bleeding in dissecting the attachment between Sg1 and IVC [1]. Due to these anatomical characteristics, local excision of Sg1 is technically demanding and requires different surgical strategies for each individual case [2].

Despite diffusion of laparoscopic liver resection (LLR), there have been a few reports on laparoscopic Sg1 resection [3, 4]. The aim of this study was to assess the safety and feasibility of the procedure by comparing its outcomes

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to those after laparoscopic resection of anteroinferior and posterosuperior segment.

Materials and methods

Study population

From January 2000 to December 2014, all patients who underwent LLR at author's institution were identified from a prospective database, and their data were retrospectively reviewed. Patients with resection of segment 1 (Sg1 group) were compared with those with resection of anteroinferior segments (AI group: segment 3, 4b, 5, 6) or posterosuperior segments (PS group: segment 4a, 7, 8), in terms of tumor characteristics, surgical treatment, and short-term outcomes. Suitability for the laparoscopic approach was based on tumor size and location, type of planned resection, and patient comorbidities. Except unusual cases with limited tumor abutment on IVC, direct involvement of IVC on preoperative imaging was considered as a contraindication to laparoscopic approach.

Preoperative evaluation

Preoperative investigations included blood and liver function tests, as well as routine cardiorespiratory evaluations. Computed tomography imaging of the thorax, abdomen, and pelvis was obtained routinely. In recent years, magnetic resonance imaging of the liver was routinely performed. No specific evaluation was required for Sg1 resection, but special attention was payed on contact between the tumor and IVC.

Surgical procedures

The surgical technique of LLR, including the positioning of the trocars, has been previously described [5, 6]. Intra-abdominal pressure was maintained at 12 mmHg. Liver resectability was routinely confirmed by intra-operative ultrasonography [7]. The gastrohepatic ligament is divided to approach segment I, preserving the accessory left hepatic artery. The hepatoduodenal ligament is dissected posteriorly, and the portal pedicles going toward segment I are identified, dissected free, and divided. The caudate lobe is mobilized from the left side and also along the anterior aspect of the IVC, dividing the short hepatic veins. The hepatic veins are usually coagulated without any clip and/or suture. The confluence of the left and middle hepatic vein with the IVC is exposed after division of the segment I hepatic vein; the duct of Arantius is cut or preserved depending on the tumor location. Then, the liver parenchyma is dissected from the caudal side toward the IVC, exposing the posterior aspect of the middle hepatic vein [8]. For all procedures, tissue dissection and hemostasis were performed using an ultrasonic dissector, such as the Harmonic scalpel (Ethicon Endo-Surgery, Inc., Cincinnati, OH) or, more recently, the Thunderbeat® (Olympus Co, Tokyo); Gayet bipolar forceps (MicroFrance CEV134, Medtronic, Minneapolis, MN) provided retraction and rescue hemostasis. All intra-operative parameters, including type and duration of vascular clamping, blood loss with subsequent intraoperative blood transfusion, and duration of surgery, were recorded. The overall surgical policy was to attempt radical anatomic or wedge resection, sparing the greatest amount of liver parenchyma feasible.

Postoperative outcomes

Postoperative complications after LLR were stratified according to the Dindo–Clavien classification, and major complications were defined as a grade $\geq$ III [9]. If a patient had two or more complications, the most severe was taken in account. Liver-specific complications were detailed as follows: Liver failure was defined according to the "50–50 criteria" on postoperative day 5 [10], ascites was defined as abdominal drainage output of >10 ml per kg per day after postoperative day 3, and biliary leakage was defined as a bilirubin concentration in the drainage fluid of more than threefold that in serum [11]. Complications and operative mortality were considered if they occurred within 90 days of surgery or at any time during the postoperative hospital stay.

Statistical analysis

Patient baseline characteristics were expressed as mean (SD) for continuous data and numbers with percentages for categorical data. Preoperative, operative, and postoperative characteristics were compared. Chi-square test was used to identify differences in categorical variables, and ANOVA was used to compare differences in categorical variables. Cumulative overall survival rates were determined using the Kaplan–Meier method and compared using the log-rank test. All statistical analyses were performed using SPSS for Windows version 21.0 (SPSS Inc.), and statistical significance was accepted at the 0.05 level.

Results

Preoperative characteristics

Of 233 patients included in this study cohort, there were 15, 151, and 67 patients in the Sg1, AI, and PS groups, respectively. Preoperative characteristics of these patients

are detailed in Table 1. The three groups did not differ significantly in terms of demographics and tumor characteristics. The rate of previous abdominal surgery was significantly higher in PS group (73.1 %) compared with Sg1 (46.6 %) and AI groups (48.3 %) ($p = 0.002$). Tumor characteristics including indication and tumor number were similar in each group, and tumor diameter was also similar in each group: Sg1 group (19.5 mm), AI group (20.0 mm), and PS group (25.0 mm), respectively ($p = 0.139$). There was no difference in the proportion of patients having previous hepatectomy (13.3 vs. 14.6 vs. 23.9 %, $p = 0.220$).

Intra-operative characteristics

The number of patients who underwent anatomical liver resection was 3 (20.0 %) in the Sg1 group, 26 (17.2 %) in the AI group, and 20 (29.9 %) in the PS group ($p = 0.590$). There was no difference in the extra-hepatic procedures performed. No patient required vascular reconstruction. Operative time was shorter in the Sg1 (150 min) and AI group (135 min) compared with PS group (180 min) ($p = 0.021$). Median blood loss was larger in the PS group (140 ml, range 0–1500 ml) compared with Sg1 group (75 ml, range 0–500 ml) and AI group (10 ml, range 0–1100 ml) ($p = 0.001$) (Table 2). There was no significant difference in use of intra-operative transfusion. Two patients required conversion in the PS group (3.0 %) and none in Sg1 and AI groups. Resection margins were clear in all Sg1 and PS group patients, whereas two (1.3 %) patients had R1 margins in AI group ($p = 0.586$).

Postoperative outcomes

There was no mortality in the three groups. Three (20.0 %) patients in Sg1 group experienced postoperative

Table 1 Preoperative characteristics

	N (%)			*p* value
	Sg1 group ($N = 15$)	AI group ($N = 151$)	PS group ($N = 67$)	
Age, year, mean ± SD	64 ± 9	59 ± 15	62 ± 13	0.624
Male gender	7 (46.6)	84 (55.6)	38 (56.7)	0.944
BMI, kg/m^2, mean ± SD	25.3 ± 4.7	25.4 ± 4.4	26.2 ± 5.0	0.700
Alcohol	3 (20.0)	33 (21.9)	12 (17.9)	0.624
Smoking	3 (20.0)	22 (14.6)	11 (16.4)	0.664
Comorbidities				
Diabetes mellitus	1 (6.6)	8 (5.3)	9 (13.4)	0.109
Hypertension	3 (20.0)	27 (17.9)	17 (25.4)	0.374
Dyslipidemia	2 (13.3)	23 (15.2)	10 (14.9)	0.760
Ischemic heart disease	0 (0)	10 (6.6)	4 (6.0)	0.632
COPD	1 (6.6)	4 (2.6)	2 (3.0)	0.601
Preoperative chemotherapy	3 (20.0)	41 (27.2)	11 (16.4)	0.236
Viral status				
HBV	0	1 (0.7)	1 (1.5)	0.776
HCV	0	2 (1.3)	1 (1.5)	0.907
Diagnosis				
CRLM	10 (66.6)	93 (61.6)	43 (64.2)	0.168
Other metastases	2 (13.3)	25 (16.6)	9 (13.4)	0.514
HCC	1 (6.6)	9 (6.0)	7 (10.4)	0.465
Cholangiocarcinoma	0 (0)	4 (2.6)	0 (0)	0.349
Benign disease	2 (13.3)	20 (13.2)	8 (11.9)	0.187
Previous abdominal surgery	7 (46.6)	73 (48.3)	49 (73.1)	0.002
Previous hepatectomy	2 (13.3)	22 (14.6)	16 (23.9)	0.220
Tumor size, mm, median (range)	19.5 (2–50)	20.0 (5–160)	25.0 (8–140)	0.139
Tumor number, median (range)	1.0 (1–2)	1.0 (1–4)	1.0 (1–4)	0.102

BMI body mass index, *COPD*, chronic obstructive pulmonary disease, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *CRLM*, colorectal cancer liver metastases, *HCC* hepatocellular carcinoma

Table 2 Intra-operative characteristics

	N (%)			*p* value
	Sg1 group ($N = 15$)	AI group ($N = 151$)	PS group ($N = 67$)	
Surgical procedures				
Pure laparoscopy	13 (86.7)	123 (81.5)	59 (88.1)	0.492
Anatomical resection	3 (20.0)	26 (17.2)	20 (29.9)	0.590
Use of Pringle maneuver	0	2 (1.3)	0	0.590
Blood loss, ml, median (range)	75 (0–500)	10 (0–1100)	140 (0–1500)	0.001
Operative time, min, median (range)	150 (60–480)	135 (60–480)	180 (60–600)	0.021
Intraoperative transfusion	0	0	3 (4.5)	0.022
Conversion	0	0	2 (3.0)	0.082
Abdominal drainage	0	5 (3.3)	2 (3.0)	0.801

complications, 22 (14.6 %) of the AI group, and 14 (20.9 %) of the PS group ($p = 0.060$) (Table 3). The rate of major complication was significantly higher in the Sg1 group (13.3 %, $N = 2$) and PS group (11.9 %, $N = 8$) compared with AI group (4.0 %, $N = 6$) ($p = 0.042$). One Sg1 patient who underwent combined lymphadenectomy developed pancreatic fistula, and this patient needed reoperation for management of this complication. Three patients in AI group and one patient in PS group required reoperation for a complication in relation to simultaneous colorectal resection. Bile leakage was observed in one patient in the AI group (0.7 %) and two PS group patients (3.0 %) ($p = 0.218$), managed by abdominal drainage. Biliary stenosis was occurred in one patient in the Sg1 group (6.6 %), successfully treated by endoscopic stenting. The length of hospital stay was significantly longer in Sg1 group (8.0 ± 6.5 days) and PS group (8.3 ± 7.3 days) compared with AI group (6.7 ± 5.1 days) ($p = 0.008$).

As shown in Table 4, Sg1 resection was not identified as an independent factor associated with postoperative major morbidity unlike PS resection. Based on multivariate analysis, COPD was found to be an independent predictor for major complication.

Discussion

As a result of this unique anatomical location, caudate lobe resection is technically challenging, because it is easy to damage the bile ducts and an error in dissecting the posterior part of the caudate lobe can cause uncontrolled bleeding from the IVC [12]. However, precise anatomical knowledge of the caudate segment, improvements in perioperative care, and refined surgical technique for caudate lobectomy in open surgery have resulted in more widespread use of this procedure. Until recently, the most favorable locations for LLR have been the peripheral liver segments [13]. However, the limitations associated with the procedure have gradually diminished with the accumulation of surgical experience in LLR. Although the reports are limited, LLR has been shown to be a feasible option for lesions located in the posterior and superior segments [14–16]. In laparoscopic view, the surgical field is visualized and accessed from the caudal side to the cranial side using a laparoscope, known as the 'caudal approach.' Thus, laparoscopic approach for caudate lobe has advantage of easy access to this location compared with approach for cranial side, such as posterosuperior segments [17–19]. However, the Sg1 is close to the liver hilum, major hepatic veins, and IVC, and is still considered theoretically as a contraindication for laparoscopic approach. Indeed, parenchymal transection near these major vessels poses greater risk of injury, and once such an injury occurs, the complications may be difficult to control laparoscopically.

The present study represents the first series reporting the results of laparoscopic Sg1 resection and the analysis compared with other lesions. Indeed, this study suggests that LLR can be safely performed for Sg1 tumors without open conversion or mortality. When compared with AI and PS groups, the Sg1 group showed a similar operative time, a significant reduction in blood loss, and a similar rate of intraoperative transfusion.

Although the danger in resection of the caudate lobe may arise from massive bleeding from the anterior part of the IVC and posterior part of the middle hepatic vein, laparoscopy has the significant advantages of providing excellent view and access to these parts behind the liver by 'caudal approach' [17–19]. Indeed, the laparoscopic approach allows precise dissection upward along the IVC. At this level, short hepatic veins for Sg1 are meticulously coagulated with the bipolar forceps and then divided rather

Table 3 Comparisons between postoperative outcomes

	N (%)			*p* value
	Sg1 group ($N = 15$)	AI group ($N = 151$)	PS group ($N = 67$)	
Postoperative mortality	0	0	0	–
Overall complication[a]	3 (20.0)	22 (14.6)	14 (20.9)	0.060
Infectious complications	2 (13.3)	7 (4.6)	8 (11.9)	0.021
Major complication[a]	2 (13.3)	6 (4.0)	8 (11.9)	0.042
Overall complication				
Liver-specific complication				
Biliary leakage	0	1 (0.7)	2 (3.0)	0.336
Intra-abdominal abscess	0	4 (2.6)	5 (7.5)	0.172
Biliary stenosis	1 (6.6)	0	0	0.001
Postoperative bleeding	0	0	1 (1.5)	0.293
Pancreatic fistula	1 (6.6)	0	0	0.001
Pulmonary complication	0	1 (0.7)	1 (1.5)	0.487
Pleural effusion	0	1 (0.7)	2 (3.0)	0.344
Ileus	1 (6.6)	4 (2.6)	0	0.172
Anastomotic leakage	0	1 (0.7)	0	0.766
General complication	0	10 (6.6)	3 (4.5)	0.542
Postoperative major complication[a]				
Liver-specific complication				
Biliary leakage	0	1 (0.7)	2 (3.0)	0.336
Intra-abdominal abscess	0	2 (1.3)	3 (4.5)	0.172
Biliary stenosis	1 (6.6)	0	0	0.001
Postoperative bleeding	0	0	1 (1.5)	0.293
Pancreatic fistula	1 (6.6)	0	0	0.001
Pulmonary complication	0	0	1 (1.5)	0.293
Pleural effusion	0	0	1 (1.5)	0.293
Anastomotic leakage	0	1 (0.7)	0	0.766
Stenosis of stomach	0	1 (0.7)	0	0.766
Ileus	0	1 (0.7)	0	0.766
Reoperation	1 (6.6)	3 (2.0)	1 (1.5)	0.364
Length of hospital stay, days, mean ± SD	8.0 ± 6.5	6.7 ± 5.1	8.3 ± 7.3	0.008

[a] Postoperative complications were stratified according to the Clavien–Dindo classification, which defines major complications by grade III or more

than clipped. We believe that clips, even locked clips, could easily slip when applied on very short veins. The posterior part of the middle hepatic vein is more inaccessible. Improved laparoscopic vision (particularly via three-dimensional camera) and the flexibility of the camera further facilitate meticulous dissection of the liver parenchyma even in the narrow surgical field at the front of the IVC and behind the liver [20].

Additionally, postoperative complications were comparable in the three groups. However, we observed nonsignificant higher rates of infectious complications and reoperation after Sg1 resection. This absence of significance may be explained by the limited sample size. However, even though Sg1 resection is scarce uncommon, we observed complications could be assigned specifically to Sg1 resection; this was the case of a postoperative biliary stricture developed by a patient six weeks after caudate lobectomy and successfully treated by biliary stenting. Indeed, we have to keep in mind that biliary drainage for Sg1 includes small tributaries to the right but occurs predominantly through the left hepatic duct [21]. Postoperative morbidity after Sg1 resection is similar to those observed after PS resection and higher than those observed after AI resection. Therefore, we should consider Sg1 as arduous location for laparoscopic approach.

Table 4 Logistic regression analysis for the risk of major complication

Risk factors	Variables	Univariate analysis			Multivariate analysis		
		p value	Odds ratio	95 % CI	*p* value	Odds ratio	95 % CI
Age (years)	≤65 versus >65	0.134	2.824	0.727–10.973			
Gender	Male versus Female	0.597	0.708	0.256–1.962			
BMI (kg/m^2)	≤30 versus >30	0.803	1.333	0.139–12.758			
Preoperative complication							
Diabetes mellitus	Negative versus positive	0.808	0.773	0.096–6.209			
Hypertension	Negative versus positive	0.897	0.918	0.250–3.364			
Dyslipidemia	Negative versus positive	0.338	0.366	0.047–2.863			
Ischemic heart disease	Negative versus positive	0.999	0.000	–			
COPD	Negative versus positive	0.043	5.943	1.057–33.415	0.033	7.319	1.178–45.480
Preoperative chemotherapy	Negative versus positive	0.128	2.209	0.795–6.136			
Previous abdominal surgery	Negative versus positive	0.129	2.462	0.769–7.878			
Previous hepatectomy	Negative versus positive	0.249	0.299	0.038–2.333			
Tumor size (mm)	≤30 versus >30	0.428	0.987	0.956–1.019			
Anatomical resection	negative versus positive	0.331	1.734	0.572–5.256			
Operation time (min)	≤300 versus >300	0.414	1.003	0.996–1.009			
Blood loss (ml)	≤500 versus >500	0.055	1.001	1.000–1.003	0.221	1.001	0.999–1.003
Sg1 group	Negative versus positive	0.233	0.377	0.076–1.871			
PS group	Negative versus positive	0.063	0.378	0.136–1.054	0.076	0.334	0.100–1.122

BMI body mass index, *COPD* chronic obstructive pulmonary disease

Another concern about the application of LLR for tumors located in Sg1 is the ability to obtain a safe resection margin. Indeed, LLR for tumors close to both the hilum and the major hepatic veins are technically challenging procedures because it may be difficult to obtain adequate surgical margins, even in open liver resection [13]. The present study emphasizes that en bloc complete caudate lobectomy involving the caudate lobe is not appropriate in all cases since all 15 patients who underwent Sg1 resection had clear surgical margins. In addition, laparoscopic ultrasound should be widely used during the procedure in order to provide a precise evaluation of tumor location and its relationship with the adjacent vascular structures [7].

The limitations of the present series include both the limited number of patients who underwent Sg1 resection and the relative heterogeneity of the tumors. Additionally, patients were highly selected given only one had cirrhosis and all tumor diameters were under 3 cm. Even though it would have been ideal to compare intra- and postoperative outcomes between laparoscopic and open Sg1 resection, our institute has high volume number of LLR; the total number of Sg1 liver resection is very low. We assume that it is a limitation of this study. Furthermore, we emphasize that surgeons should have experienced technique of LLR when considering laparoscopic Sg1 resection.

In conclusion, this study suggests that the laparoscopic approach is a feasible and curative surgical option for resection of tumors located in the caudate lobe with acceptable operative time, postoperative outcomes, and tumor-free margins in selected patients.

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Compliance with ethical standards

Disclosures Kenichiro Araki, David Fuks, Takeo Nomi, Satoshi Ogiso, Ruben R Lozano, Hiroyuki Kuwano, and Brice Gayet have no conflict of interest. Brice Gayet is consultant for Olympus.

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High Expression of Karyopherin-α2 and Stathmin 1 is Associated With Proliferation Potency and Transformation in the Bile Duct and Gall Bladder Epithelia in the Cases of Pancreaticobiliary Maljunction

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Backgrounds and Objectives: Pancreaticobiliary maljunction (PBM) may be associated with an increased frequency of gall bladder cancer with no bile duct dilation. Karyopherin-α2 (KPNA2) and stathmin 1 (STMN1) were reported to play important roles in carcinogenesis and cancer progression.

Methods: Fifteen patients with PBM who underwent surgical resection between 1999 and 2014 were included in this study. Using immunohistochemistry, we investigated the expression of p53, Ki-67, KPNA2, and STMN1 in normal biliary tract epithelium, hyperplastic epithelium, and cholangiocarcinoma (CC) tissues.

Results: Nuclear expression of KPNA2, p53, and Ki-67 expression was detected in hyperplastic epithelium and CC tissues. High KPNA2 expression was significantly associated with gender ($P = 0.04$), p53 nuclear accumulation ($P = 0.00435$), and Ki-67 expression ($P = 0.0443$) in the gall bladder and bile duct of PBM. On the other hand, STMN1 was only expressed in CC tissues and was not observed in normal bile duct and hyperplastic epithelia.

Conclusions: KPNA2 might be a useful marker of hyperplasia, dysplasia, and carcinogenicity in patients with PBM. STMN1 evaluation might be a cancer-specific marker for CC patients with PBM similar as that for other cancers.

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KEY WORDS: **inportin; stathmin 1; Op18; biliary tract cancer; PBM**

INTRODUCTION

Pancreaticobiliary maljunction (PBM) is an innate anomaly in which the pancreatic and bile ducts meet outside the duodenal wall [1,2]. Because of the incompetent union at the sphincter muscle of Oddi, this anomaly causes regurgitation of bile and pancreatic juice. PBM has been associated with an increased frequency of gall bladder cancer with no bile duct dilation [3]. With the continued stimulation of chronic inflammation, the gall bladder mucosa may change into a hyperplastic epithelium. The hyperplasia–dysplasia-carcinoma sequence has been reported as a mechanism of biliary carcinogenesis in PBM [4,5]. Studies have reported that p53 and Ki-67 expression was altered and dependent on the progression of the sequence [6,7]. Because cholangiocarcinomas (CC) often undergo local progress permeation infiltration and metastases, radical excision may be difficult; therefore, it has a poor prognosis [8,9]. It is important to develop a diagnostic biomarker to predict lethal CC progression in patients with PBM. Moreover, such a marker might be a promising molecular candidate for targeting carcinogenesis in the biliary systems of patients with PBM.

Stathmin 1 (STMN1) is one of the cytosolic phosphoproteins that regulates microtubule dynamics by promoting microtubule destabilization [10]; therefore, it is a microtubule destabilizer. STMN1 plays an important role in carcinogenesis and cancer progression [11]. Many types of human malignancies have expressed higher STMN1 levels than non-cancerous tissues, and this expression has been associated with cancer progression and therapeutic resistance, particularly against microtubule agents. In addition, STMN1 is also known as oncoprotein 18 (op18). Previously, many researchers have reported the clinical significance of STMN1 expression in malignant tumors, including breast cancer [12,13], prostate cancer [14], cervical cancer [15], malignant mesothelioma [16], gastric cancer [17,18], hepatocellular carcinoma [19], oral squamous cell carcinoma, endometrial cancer [20], colorectal cancer [21], and upper urinary

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tract urothelial carcinoma [22]. In particular, we first presented the clinical significance of STMN1 in sporadic CC [23]. These studies suggested that STMN1 could be a potential target for diagnosis and treatment even in CC with PBM; however, the exact significance of STMN1 in biliary systems, including those with CC and PBM, has not yet been determined.

Recent studies have identified high expression levels of karyopherin-α2 (KPNA2) as a marker of poor prognosis in a variety of cancer types [24], including breast cancer [25], malignant melanoma [26], esophageal cancer [27], lung cancer [28], ovarian cancer [29], prostate cancer [30], brain cancer [31], and hepatocellular carcinoma [32]. KPNA2 has been reported to transport several macromolecules larger than 50 KDa or other complex proteins from the cytoplasm into the nucleus. The cargo proteins transported by KPNA2 include several cancer-related proteins, including p53, myc, E2F, MRN complex, and the STAT family. Among these proteins, p53, myc, STAT, and the MRN complex have been suggested to play important roles in carcinogenesis, proliferation, and the resistance to chemotherapy in bile duct cancer [33–36]. However, few previous studies have addressed whether KPNA2 expression is related to the progression to hyperplasia and carcinogenesis in the bile duct and gall bladder of patients with PBM.

The purpose of this research was to clarify the significance of KPNA2 and STMN1 expression in the biliary tracts of patients with PBM, including the normal biliary epithelium, hyperplastic epithelium, and cancerous tissue using immunohistochemistry. Moreover, we examined the changes in the expression of KPNA2 and STMN1, the proliferation marker Ki-67, and transformation-related marker p53 in the hyperplasia–dysplasia-carcinoma sequence of these samples.

MATERIALS AND METHODS

Patients and Samples

Fifteen patients with PBM who underwent surgical resection in our department between 1999 and 2014 were included in this study. There were four males and 11 females. The mean age of patients was 48.5 years (range, 16–77). There were four patients with PBM who had cancer of the gall bladder (two cases) or bile duct (two cases). The Todani classification of the PBM cases were graded as type Ia (n = 3), type Ic (n = 3), type IVa (n = 4), type V (n = 1), and non-dilatation (n = 4), according to a previous report [3]. None of patients had received neoadjuvant chemotherapy and/or irradiation before the surgical resection. Written informed consent was obtained from all patients. This study was approved by the institutional review board at Gunma University Hospital.

Immunohistochemical Staining

A paraffin-embedded block for each of the PBM specimens was cut into 2 μm-thick sections and mounted on glass slides. Each section was deparaffinized using xylene and dehydrated in alcohol. Endogenous peroxidase was inhibited using 0.3% H_2O_2/methanol for 30 min at room temperature. The sections were soaked in heated water with 0.5% Immunosaver (Nishin EM, Tokyo, Japan) at 98°C for 45 min. Non-specific antigens were blocked by Protein Block Serum-Free (DAKO, Glostrup, Denmark) at room temperature for 30 min. The sections were then incubated with primary antibodies against KPNA2 (Abcam, Tokyo, Japan, Rabbit anti-KPNA2 polyclonal antibody, 1: 400), STMN1 (Santa Cruz Biotechnology, Santa Cruz, CA, Mouse monoclonal anti-STMN1 antibody, 1: 100), p53 (DAKO, DO-7, Monoclonal Mouse Anti-Human p53, 1:100), and Ki67 (DAKO, MIB-1, Monoclonal Mouse Anti-Human Ki-67, 1: 200) for 24 hr at 4°C. After washing with phosphate buffered saline, the Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) was applied for visualizing the primary antibody and incubated for 45 min. The chromogen 3,3′-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution containing 0.005% H_2O_2 in 50 mM of ammonium acetate–citrate acid buffer (pH 6.0). Finally, counterstaining of the nucleus was performed using Mayer's Hematoxylin solution. We adopted a negative control by replacing the primary antibody with phosphate buffered saline in 0.1% bovine serum albumin and confirmed no detectable staining with the negative control.

Assessment of KPNA2, STMN1, p53, and Ki-67 Expression

We evaluated the cytoplasmic staining of STMN1 in the normal epithelium, hyperplastic epithelium, and cancerous tissue of patients with PBM. KPNA2-positive cells were defined as those with a brown-stained nucleus, regardless of staining intensity. Three staining patterns were identified: positive, positive cells in most of the lesion; moderate, positive cells aggregated in a focal area of the lesion and small numbers of isolated positive cells scattered throughout the lesion; and negative. Cytoplasmic STMN1 was scored as follows: 0, no staining; 1+, 1–10%; 2+, 11–50%; and 3+, 51–100%. The optimal cut-off point was defined as follows: grades 0, 1, and 2 were considered negative, and grade 3 was designated as positive. p53-positive cells were defined as those with a brown-stained nucleus, regardless of the staining intensity. The following four staining patterns were identified: positive cells in most of the lesion (diffuse), positive cells aggregated in a focal area of the lesion (nested), small numbers of isolated positive cells scattered throughout the lesion (scattered), and negative. Positive p53 protein expression was defined as either a diffuse or nested pattern and negative p53 protein expression was defined as a scattered pattern throughout the lesion or negative as previously described [37,38]. For the evaluation of Ki-67 expression, a positive cell frequency of more than 10% was assessed as positive.

Statistical Analysis

Data for continuous variables were expressed as mean ± standard deviation (SD). Associations between KPNA2 expression and clinicopathological characteristics were analyzed using the chi-square and Mann–Whitney U tests. All differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using the JMP software package (SAS Institute Inc., Cary, NC).

RESULTS

Expression Analysis of KPNA2, STMN1, p53, and Ki-67 in the Gall Bladder and Bile Duct of Patients With PBM by Immunohistochemistry

Immunohistochemistry analysis showed that KPNA2 was expressed in cell nuclei. Therefore, nuclear KPNA2 expression was evaluated in the gall bladder or bile duct of 15 patients with PBM. In all cases, nuclear KPNA2 expression was not observed in normal gall bladder or bile duct epithelium (Fig. 1A) and (Table I). However, the expression level of KPNA2 increased during hyperplasia (66.7%, 10/15) and in cancerous tissues (100%, 5/5) (Fig. 1A) and (Table I). We investigated the correlation of KPNA2, p53, and Ki-67 expressions by immunohistochemistry in representative sections of normal, hyperplastic, and cancerous tissues from identical cases. High KPNA2 expression sections demonstrated the enhanced expression of p53 and Ki-67 in hyperplastic and cancerous tissues (Fig. 1B,C).

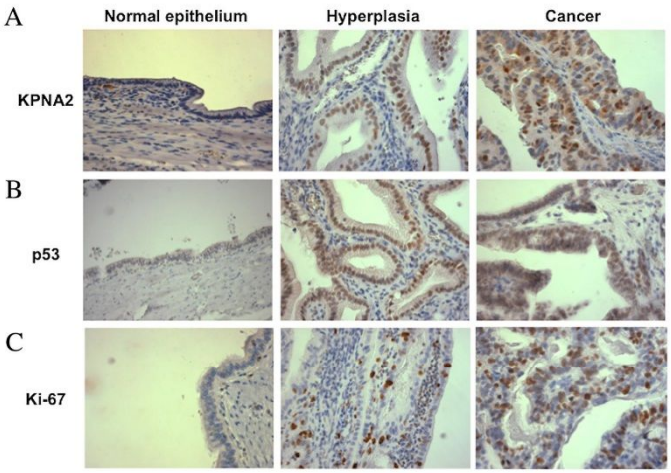

Fig. 1. KPNA2, p53, and Ki-67 expression in representative tissues of patients with PBM. (**A**) The expression of nuclear KPNA2 in representative normal epithelium, hyperplastic tissue, and cancerous tissue in the biliary tract of patients with PBM (original magnification: ×20, ×40, and ×40, respectively). (**B**) The expression of nuclear p53 in representative normal epithelium, hyperplastic tissue, and cancerous tissue in the biliary tract of patients with PBM (original magnification: ×20, ×40, and ×40, respectively). (**C**) The expression of nuclear Ki-67 in representative normal epithelium, hyperplastic tissue, and cancerous tissue in the biliary tract of patients with PBM (original magnification: ×20, ×40, and ×40, respectively).

STMN1 was expressed only in the CC tissues and was not observed in the normal bile duct or hyperplastic epithelium in all cases (Fig. 2A). STMN1 and p53 expression level in CC tissues of patients with PBM was 80%. STMN1 and p53 expression in CC tissues had the same frequency (Table I). p53 expression level in the hyperplastic epithelium of patients with PBM was 40%. Also, p53 expression was not observed in the normal epithelium (Fig. 2B) and (Table I). Furthermore, Ki-67 expression level in the hyperplastic epithelium of patients with PBM was 26.7% (Fig. 2C) and (Table II). p53 and Ki-67 expression was induced depending on the progression of the hyperplasia-carcinoma sequence in patient with PBM.

Relationships Between the Expression of KPNA2 and Clinicopathological Factors in the Biliary Tract of Patients With PBM

We divided our 15 samples into three groups according to the intensity of nuclear KPNA2 staining in the gall bladder or bile duct with hyperplasia. The correlations between KPNA2, p53, and Ki-67 expressions and clinicopathological characteristics are shown in Table II. High KPNA2 expression was significantly associated with gender ($P = 0.04$), p53 nuclear accumulation ($P = 0.0435$), and Ki-67 expression ($P = 0.0443$). However, there were no significant differences with respect to age, dilatation, or cancer coexistence.

TABLE I. p53, Ki67, KPNA2, and STMN1 Expression in the Biliary System of CC Tissues of Patients

	Normal biliary epithelium* (two cases without normal biliary epithelium)	Hyperplasia (15 PBM cases)	Cancers (five lesions from four cancer cases with PBM)
p53	0% (0/13)	40% (6/15)	80% (4/5)
Ki-67	0% (0/13)	26.7% (4/15)	80% (4/5)
KPNA2	0% (0/13)	66.7% (10/15)	100% (5/5)
STMN1	0% (0/13)	0% (0/15)	80% (4/5)

*Two cases without nornal biliary epithelium.

Analysis of STMN1 Expression in CC Tissues of Patients With PBM Using Immunohistochemistry

STMN1 was highly expressed in the cytoplasm of the cancerous tissue and not expressed in the cytoplasm of the normal or hyperplastic epithelium (Fig. 3A,B). In addition, STMN1 was not expressed in the adenoma tissue. On the other hand, STMN1 was expressed in the carcinoma in situ (Fig. 3C). STMN1 was expressed only in the cancerous tissue. In particular, CC cells in the invasion front were expressing high levels of STMN1 compared with that expressed by the main CC tumors (Fig. 3D).

DISCUSSION

In this study, we clarified that STMN1 expression was higher in only the CC lesions derived from patients with PBM and that KPNA2, Ki67, and p53 expression was induced in the hyperplastic epithelium and cancerous tissues of patients with PMB depending on the progression of the hyperplasia-carcinoma sequence.

Progression from a hyperplastic state to a cancer lesion in the biliary tract is important and represents a lethal problem in patients with PBM, including many young individuals because of a congenital anatomical anomaly. The overexpression of p53 is reported to be associated with carcinogenesis in the mucosal epithelia of the biliary system in patients with PBM [39]. The expression rate of p53 in cancer, carcinoma in situ, and dysplasia was 65%, 44.7%, and 2.4%, respectively [40]. In this

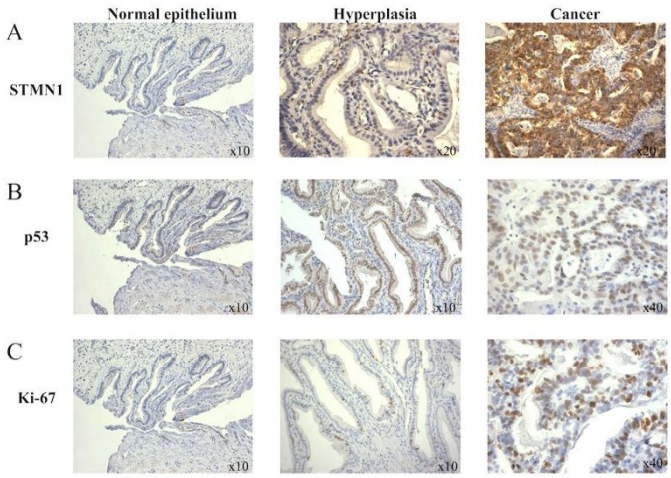

Fig. 2. STMN1, p53, and Ki-67 expression in representative tissues of patients with PBM. (**A**) Cytoplasmic STMN1 expression in the representative normal epithelium, hyperplastic epithelium, and cancerous tissues of the biliary tract of patients with PMB (original magnification: ×10, ×20, and ×20, respectively). (**B**) Nuclear p53 expression in the representative normal epithelium, hyperplastic epithelium, and cancerous tissues of the biliary tract of patients with PMB (original magnification: ×10, ×10, and ×40, respectively). (**C**) Nuclear Ki-67 expression in the representative normal epithelium, hyperplastic epithelium, and cancerous tissues of the biliary tract of patients with PMB (original magnification: ×10, ×10, and ×40, respectively).

study, the positive detection rate for p53 was 40% and 80% for hyperplasia and cancers, respectively. This positive rate is slightly higher than that cited in previous reports [40]. The nuclear KPNA2 accumulation in the biliary tract mucosa of patients with PMB was significantly related to the expression of transformation-related protein p53 and the proliferation marker Ki-67, and such accumulation was detected in all cancerous tissues (100%, 5/5 cancer lesions from four patients with PBM). KPNA2 might overcome the existing hyperplasia-carcinogenesis marker p53 in patients with PBM.

TABLE II. Relationship Between the Expression of KPNA2, p53, and Ki-67 and Clinicopathological Factors in the Biliary Tract of PBM Patients

	KPNA2 expression in hyperplasia of PBM			
Factor	Negative, n = 5	Moderate, n = 3	Positive, n = 7	*P*-value
Age (yr) (mean ± SD)	55.6 + 12.9	22.7 + 9.86	54.6 + 18.8	0.0728
Gender				
Male	0	0	4	0.04*
Female	5	3	3	
p53 accumulation				
Absent	5	2	2	0.0435*
Present	0	1	5	
Ki-67 expression				
Low <10%	5	3	3	0.0443*
High >10%	0	0	4	
Dilatatoin				
Absent	2	0	2	0.46
Present	3	3	5	
Cancer coexistence				
Absent	4	3	3	0.51
Gall bladder	0	0	2	
Bile duct	1	0	1	
Pancres	0	0	1	

*$P < 0.05$.

Biliary carcinogenesis secondary to chronic inflammation has been a clinically significant problem, and novel markers for diagnosis are required. In this study, STMN1 was not expressed in non-cancerous portions of the tissue and in the hyperplastic epithelium and was very highly expressed in the cancerous part. STMN1 has been reported to be expressed in higher levels in various cancers compared with those normal tissues, and has been associated with poor prognosis, cancer progression, and taxane anticancer agent resistance in idiopathic CC [23]. These data were consistent with previous studies from many researchers. STMN1 might be a promising candidate for targeted therapy and for controlling the progression of refractory CCs with or without PBM.

In a preclinical study, the measurement of KPNA2 and STMN1 levels in the serum of cancer patients by enzyme-linked immunosorbent assay suggested that a high expression of these proteins represents a marker for lung and urinary cancer [28,41,42]. From these observations, KPNA2 and SMTN1 testing with liquid samples from preoperative or prediagnostic patients with PBM might represent a new marker to predict carcinogenesis in the biliary tract of patients with PBM.

Several factors have been reported as STMN1 regulators, including p53, p27, and the PI3K/AKT pathway [43,44]. These factors may be related to the activation of mitogen-activated protein kinase (MAPKs) and signal transducer and activator of transcription 3 (STAT3) in CC [45–47]. From these data, it was suggested that STMN1 regulation by these factor might be important in CC carcinogenesis in part via activation of STAT3 and MAPKs. On the other hand, STMN1 expression is induced by p53 mutation [48]. STMN1 and p53 expression in CC patients with PBM was 80%. STMN1 and p53 expression had the same frequency. However, STMN1 expression was not detected in the hyperplastic epithelium with p53 accumulation;

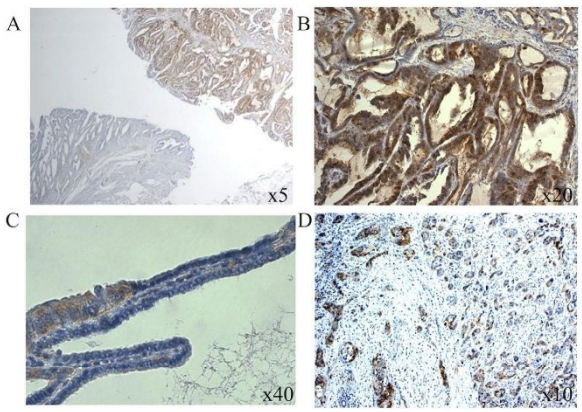

Fig. 3. Immunohistochemical staining of stathmin 1 (STMN1) in cholangiocarcinoma (CC) tissues of patients with PBM. (**A**) STMN1 expression in hyperplastic epithelium (left side) and CC tissues (right side) (original magnification ×5). TMN1 was highly expressed in the cytoplasm of the cancerous tissue. STMN1 was not expressed in the cytoplasm of the normal epithelium and hyperplastic mucosa. (**B**) A representative imaging of a case of high STMN1 expression in CC tissue (original magnification ×20). Part B is a magnified image of Part A. (**C**) STMN1 expression in the marginal region between carcinoma in situ and the adjacent non-cancerous tissue (original magnification ×40). STMN1 was not expressed in the adenoma. On the other hand, STMN1 was expressed in the carcinoma in situ tissue. (**D**) STMN1 expression in the CC tissues between invasion line (left side) and the main tumors (right side) (original magnification ×10). CC cells in invasion line expressed high levels of STMN1 compared with the main CC tumors.

therefore, p53 may not be the main adjustment mechanism for STMN1 in CC patients with PBM.

Importin α proteins, including KPNA2, have many important cellular functions in both cancerous and non-cancerous tissues because fundamental mechanisms such as proliferation and DNA repair are necessary for all types of cells [49,50]. The main importin α protein in a variety of non-cancerous adult tissues is importin α5 (KPNA1) [51]. However, in the present study, we focused on the cancer-specific importin α family member KPNA2, which is over-expressed in only cancerous and stem cells [49]. KPNA2 expression is induced by the activation of the KPNA2 promoter by E2F [52], which is expressed in several cancerous and stem cells [53,54]. E2F is reported to be one of the KPNA2 cargo proteins, and the nuclear translocation of E2F by KPNA2 is needed to activate transcriptional capability. In this study, it is suggested that a positive feedback loop involving KPNA2 and E2F is an important regulatory mechanism for KPNA2 in the biliary system and cancers in patients with PBM.

Therefore, KPNA2 expression in the biliary tract mucosa of patients with PBM was correlated to the expression of p53 and Ki67. KPNA2 is therefore expected to be a useful marker of hyperplasia, dysplasia, and carcinogenicity. STMN1 expression contributed to carcinogenesis in the biliary systems of patients with PBM. STMN1 evaluation might be a cancer-specific marker for CC patients with PBM similar as that for other cancers. Moreover, many researchers have been interested in the possibility of KPNA2 and STMN1 as a therapeutic target against refractory cancers. Targeting strategy against these proteins may be a promising molecular target for controlling the progression of CC with PBM.

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