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Prognosis and Predictors of Surgical Complications in Hepatocellular Carcinoma Patients With or Without Cirrhosis after Hepatectomy

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Abstract
Background Although poor liver function is associated with a high morbidity rate and poor prognosis in hepatocellular carcinoma (HCC) patients, the exact effects of liver pathology on the surgical outcomes of HCC patients are poorly understood. The purpose of this study was to assess how the liver pathology of HCC patients affects their prognosis and complications rate after liver resection.
Methods Between January 2006 and November 2010, 149 consecutive hepatocellular carcinoma patients, including 79 noncirrhosis patients and 70 cirrhosis patients, were enrolled in this study.
Results Among the noncirrhotic patients, operative time, fresh frozen plasma (FFP) transfusion requirement, tumor size, and serum retinol binding protein (RBP) levels were significantly higher in the complications group than in the complications-free groups. On the other hand, in the cirrhotic patients the prothrombin time (PT) and indocyanine green retention value at 15 min ($ICGR_{15}$) of the complications group were significantly lower and higher, respectively, than those of the complications-free group. In the noncirrhotic patients, recurrence-free survival and overall survival did not differ between the complications and complications-free groups. On the other hand, in the cirrhotic patients, the recurrence-free survival and overall survival of the complications-free group were significantly longer than those of the complications group.
Conclusions In the noncirrhotic patients, surgical complications had no prognostic effect, whereas they had a significant survival impact in the cirrhotic patients. The surgical strategy for HCC should be based on the patient's pathological background.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer worldwide and the third most common cause of cancer-related death [1, 2]. The optimal management strategy for HCC depends on both tumor-related factors and host liver function [3, 4]. Although the frequency of nonalcoholic steatohepatitis-related HCC has recently increased [5, 6], most HCC still develops in patients with viral hepatitis-associated liver disease [1, 2]. In the era when no effective viral therapy was available, a high incidence of recurrence after treatment was inevitable in HCC patients. Therefore, surgery for HCC tended to be avoided in patients with good liver function [7]. In addition, the high mortality rate of liver resection itself encouraged patients and doctors to select interventional therapy instead of a surgical approach.

However, liver surgery techniques have improved, and the mortality rate after liver resection was nearly zero in recent cases [8, 9]. In addition, technical developments have encouraged surgeons to select a laparoscopic approach instead of conventional open surgery [10, 11]. In liver resection for HCC, the current goal is to reduce the morbidity rate as much as possible and improve patient prognosis. Liver transplantation is considered to be the best curative approach for HCC, but liver resection should be

considered in cases in which it would be expected to achieve a good prognosis [9, 12]. Furthermore, the mortality rate of liver resection is lower than that of liver transplantation in the early stages of HCC, and among patients with good liver function the long-term prognosis of patients who undergo liver resection is comparable to that of patients that undergo liver transplantation [9].

Although poor liver function is associated with a high morbidity rate and poor prognosis in HCC patients [13, 14], the exact effects of liver pathology on the surgical outcomes of HCC patients are poorly understood. Therefore, the purpose of this study was to identify how liver pathology affected the prognosis and complications rates of consecutive HCC patients who underwent liver resection.

Patients and methods

Between January 2006 and November 2010, 149 consecutive hepatocellular carcinoma patients who underwent hepatectomy were enrolled in this study after providing informed consent. Mortality was defined as any in-hospital death that occurred within 90 days of surgery. Postoperative complications were defined and classified according to the modified Clavien classification system [15]. Briefly, Grade I complications were defined as any deviation from the normal postoperative course that did not require special treatment. Grade II complications were defined as those that required pharmacological treatment with drugs. Grade III complications were defined as those that required surgical or radiological intervention with (IIIb) or without (IIIa) general anesthesia. Grade IV complications were defined as life-threatening complications involving single (IVa) or multiple (IVb) organ dysfunction. Grade V complications were defined as those that caused the death of the patient. For grade IV or worse complications, liver failure was defined as a serum bilirubin concentration of greater than 5 mg/dl that lasted for more than 2 days. Renal dysfunction/insufficiency was defined as oliguria (<400 ml/day) combined with a sustained serum creatine level elevation of more than 1.1 mg/dl. Bleeding was diagnosed by endoscopic examination. Wound seroma/infection was defined as any wound that split open regardless of whether bacteria were detected. Ascites was defined as fluid discharge of more than 300 ml/day for more than 3 days.

We divided the patients into two groups: the noncirrhosis group (79 patients) and the cirrhosis group (70 patients). The study design conformed to the ethical guidelines of the Declaration of Helsinki, and obtained informed consent was obtained from each subject before their registration.

Assessment of clinical and operative variables

Before hepatectomy, we performed laboratory tests to assess the patients' serum levels of type IV collagen (Col), hyaluronic acid (HA), prealbumin (PreALB), retinol binding protein (RBP), hepatocyte growth factor (HGF), alpha fetoprotein (AFP), and protein induced by vitamin K absence or antagonists-II (PIVKAII), as well as their indocyanine green retention value at 15 min ($ICGR_{15}$) and ^{99}m-technetium-labeled galactosyl serum albumin (GSA) scintigraphy index (HH15, LHL15) values. Their intraoperative data and any complications that occurred during hospitalization also were recorded. Tumor size and number were assessed by pathological examinations. All laboratory tests were conducted in the early morning on the day of assessment. The model for end-stage liver disease (MELD) score of each patient was calculated using the following formula: $9.57 \times \text{Ln}$ (creatinine mg/dL) $+ 3.78 \times \text{Ln}$ (bilirubin mg/dL) $+ 11.20 \times \text{Ln}$ (PT-INR) $+ 6.43$, based on laboratory tests [16].

The Child–Pugh score with Pugh's modification was calculated as the sum of the scores for five clinical parameters [ascites (none = one point, moderate = two points, severe = three points), serum bilirubin (<2 mg/dl = one point, 2–3 mg/dl = two points, >3 mg/dl = three points), serum albumin (>3.5 g/dl = one point, 2.8–3.5 g/dl = two points, <2.8 g/dl = three points), hepatic encephalopathy (absent = one point, grade 1 or 2 = two points, grade 3 or 4 = three points), and prothrombin index [>70 % = one point, 40–70 % = two points, <40 % = three points]). Then, the patients were classified into three groups with different expected survivals according to their Child–Pugh scores (Child–Pugh A = 5–6 points, Child–Pugh B = 7–9 points, Child–Pugh C = 10 or more points) [17].

Surgical procedure

All liver resections were performed with the Pringle maneuver after more than 300 ml of intraoperative bleeding. Hepatic flow was not controlled if the intraoperative bleeding was less than 300 ml. A Cavitron ultrasonic aspirator (CUSA) was used for liver parenchymal dissection, and an argon laser beam coagulator or saline-associated monopolar electrocautery was used to achieve hemostasis. Antibiotics were administered 30 min before the laparotomy and every 3 h during the operation. All of the sutures and ties, except those used for skin closure, were absorbable (Vicryl or PDS, Johnson & Johnson Gateway, Piscataway, NJ). The periwound skin was washed with 500 ml of warm saline before skin closure. A closed-type intra-abdominal drain and a subcutaneous drain were installed for 2–3 days after the liver resection.

The operation type was classified as follows: hepatic resection (Hr) 0: partial resection including tumor enucleation; Hr S: sub-segmentectomy; Hr 1: mono-segmentectomy; Hr 2: bi-segmentectomy including right hepatectomy, left hepatectomy, and central bi-segmentectomy; and Hr 3: tri-segmentectomy.

Statistical analysis

For the statistical analyses, demographic data and perioperative laboratory test results were extracted from the clinical database, and the differences among the groups were compared using the χ^2 test followed by the post-hoc 2×2 Fisher's exact test, when necessary. Continuous variables were compared using the Mann–Whitney U test. Logistic regression analysis was used to identify the most relevant risk factors for complications. The factors affecting overall survival were assessed using the Kaplan–Meier method, with comparisons performed using the log-rank test and univariate or multivariate analyses performed using the Cox proportional hazards regression model. All calculations were performed using the StatView 5.0 software package (Abacus Concepts Inc., Berkeley, CA) or SPSS 16.0 (SPSS Inc., Chicago, IL). Receiver operating characteristic (ROC) curves, which were used to calculate the area under the ROC curve (AUC), were produced using the MedCalc software package (Ver 8.0.1.0, Mariakerke, Belgium). All results are expressed as median values (minimum value-maximum value). P values <0.05 were considered to be statistically significant.

Results

Of the 149 consecutive patients in which we performed hepatectomy for HCC, postoperative pathological liver evaluations found that 79 patients had noncirrhotic livers, and 70 patients had cirrhotic livers. The two groups displayed similar morbidity rates, and there were no significant differences in the frequencies of any of the complications included in the Clavien classification (Table 1).

Table 2 shows the clinical demographics of the noncirrhotic patients. In univariate analysis of these patients, the operative time, fresh frozen plasma (FFP) transfusion requirement, tumor size, MELD score, and γ-glutamyl transpeptidase (gGT), and retinol binding protein (RBP) levels of the complications group were found to be significantly higher than those of the complications-free group. Multivariate analysis demonstrated that all of these indicators, except gGT and the MELD score, were significantly increased in the complications group. The area under the curve (AUC) values of these indicators were all greater than 0.65 and were significantly different (Fig. 1a–d). Interactive dot diagrams were used to determine the ideal cutoff values for each parameter, which were 3.1 mg/dl for RBP (Fig. 1e), 373 min for operative time (Fig. 1f), 0 U for FFP transfusion requirement (Fig. 1g), and 5.5 cm for tumor size (Fig. 1h).

Table 1 Postoperative complications suffered by noncirrhotic and cirrhotic patients after hepatectomy for hepatocellular carcinoma

Clavien classification	Noncirrhotic ($n = 79$)		Cirrhotic ($n = 70$)	
	GI–GIII	GIV–GV	GI–GIII	IV–GV
Liver failure	1	1	1	1
Bleeding	1		2	
Bile leakage	3		1	
Respiratory distress	5		1	1
Renal dysfunction/failure	2		2	
Intra-abdominal abscess	3		2	
Wound seroma/infection	5		3	
Pleural effusion	3		5	
Ascites	3		10	
	28 events/ 22 patients		29 events/ 24 patients	

Table 3 shows the clinical demographics of the cirrhotic patients. In univariate analysis of these patients, the prothrombin time (PT), choline esterase (CholE) levels, Child–Pugh score, and MELD score of the complications group were found to be significantly higher than those of the complications-free group, whereas the indocyanine green retention value at 15 min ($ICGR_{15}$) of the complications group was significantly lower than that of the complications-free group. Of these, PT and the $ICGR_{15}$ achieved significance in the multivariate analysis. The AUC values of both of these parameters were greater than 0.65 and were significantly different (Fig. 2a, b). Interactive dot diagrams demonstrated the ideal cutoff values for each of these parameters, which were 82.8 % for PT (Fig. 2c) and 9.6 % for the $ICGR_{15}$ (Fig. 2d).

We next examined the recurrence-free survival (Fig. 3a, c) and overall survival (Fig. 3b, d) rates of the noncirrhotic (Fig. 3a, b) and cirrhotic patients (Fig. 3c, d). Among the non-cirrhotic patients, the complications and complications-free groups displayed similar recurrence-free survival and overall survival rates. On the other hand, among the cirrhotic patients, the complications-free group demonstrated significantly longer recurrence-free survival and overall survival than the complications group.

Discussion

In this study, we showed that the risk factors for perioperative complications differed between noncirrhotic

Table 2 Clinical demographics of the noncirrhotic patients who underwent initial hepatectomy for hepatocellular carcinoma ($N = 79$)

	Complications	Complications-free	Univariate	Multivariate
Etiology			0.262	
B	8	25		
C	6	20		
BC	1	0		
NBNC	7	12		
Operation			0.054	
0	5	24		
S	3	13		
1	6	11		
2	4	8		
3	4	1		
Stage			0.678	
1	1	8		
2	16	32		
3	3	10		
4	2	7		
Operative time (min)	415.6 ± 143.9	332.7 ± 134.8	0.019	0.043
Bleeding (ml)	857.8 ± 609.3	552.4 ± 1261.1	0.282	
Blood transfusion (U)	1.9 ± 3.2	0.6 ± 2.4	0.053	
FFP transfusion (U)	4.0 ± 6.1	1.1 ± 3.1	0.006	0.001
Tumor size (cm)	6.62 ± 4.06	4.22 ± 3.14	0.009	0.001
Tumor number	1.6 ± 1.2	1.6 ± 1.5	0.979	
Age (year)	67.7 ± 9.2	67.9 ± 11.2	0.931	
Height (cm)	161.9 ± 7.6	160.5 ± 7.5	0.463	
Weight (kg)	61.4 ± 10.2	58.7 ± 9.8	0.289	
BMI	23.5 ± 3.1	22.7 ± 3.2	0.307	
ALB (g/dl)	3.81 ± 0.53	3.93 ± 0.35	0.252	
Bil (mg/dl)	0.61 ± 0.34	0.72 ± 0.34	0.189	
PT (%)	92.9 ± 9.4	92.8 ± 13.5	0.979	
Plt ($\times 10^4$)	18.2 ± 6.8	17.7 ± 11.5	0.844	
AT (%)	96.9 ± 16.8	94.4 ± 17.2	0.577	
AST (IU/L)	43.4 ± 25.6	38.8 ± 39.4	0.617	
ALT (IU/L)	41.4 ± 29.7	36.4 ± 30.9	0.516	
gGT (IU/L)	117.2 ± 180.6	52.7 ± 47.1	0.022	0.056
CholE (IU/L)	245.3 ± 79.1	250.4 ± 66.1	0.776	
Col (ng/ml)	5.52 ± 2.83	4.99 ± 1.46	0.322	
HA (ng/ml)	160.1 ± 143.5	141.6 ± 207.1	0.712	
BTR	6.49 ± 2.21	6.53 ± 1.79	0.941	
ICG R15 (%)	10.1 ± 8.5	10.8 ± 6.7	0.692	
RBP (mg/dl)	4.47 ± 3.68	2.68 ± 1.13	0.005	0.024
PreALB (mg/dl)	21.3 ± 8.1	18.8 ± 6.6	0.195	
HGF (ng/ml)	0.34 ± 0.11	0.29 ± 0.13	0.199	
HH15	0.602 ± 0.062	0.584 ± 0.069	0.299	
LHL15	0.930 ± 0.027	0.933 ± 0.029	0.675	
Child–Pugh score	5.318 ± 0.477	5.281 ± 0.701	0.818	
MELD score	8.901 ± 5.314	7.402 ± 1.156	0.046	0.095

patients and cirrhotic patients who had undergone liver resection for HCC. In addition, the effects of surgical complications on postoperative recurrence-free survival and overall survival also differed among these groups. These results indicate that the pathological state of the patient's liver should be taken into account when

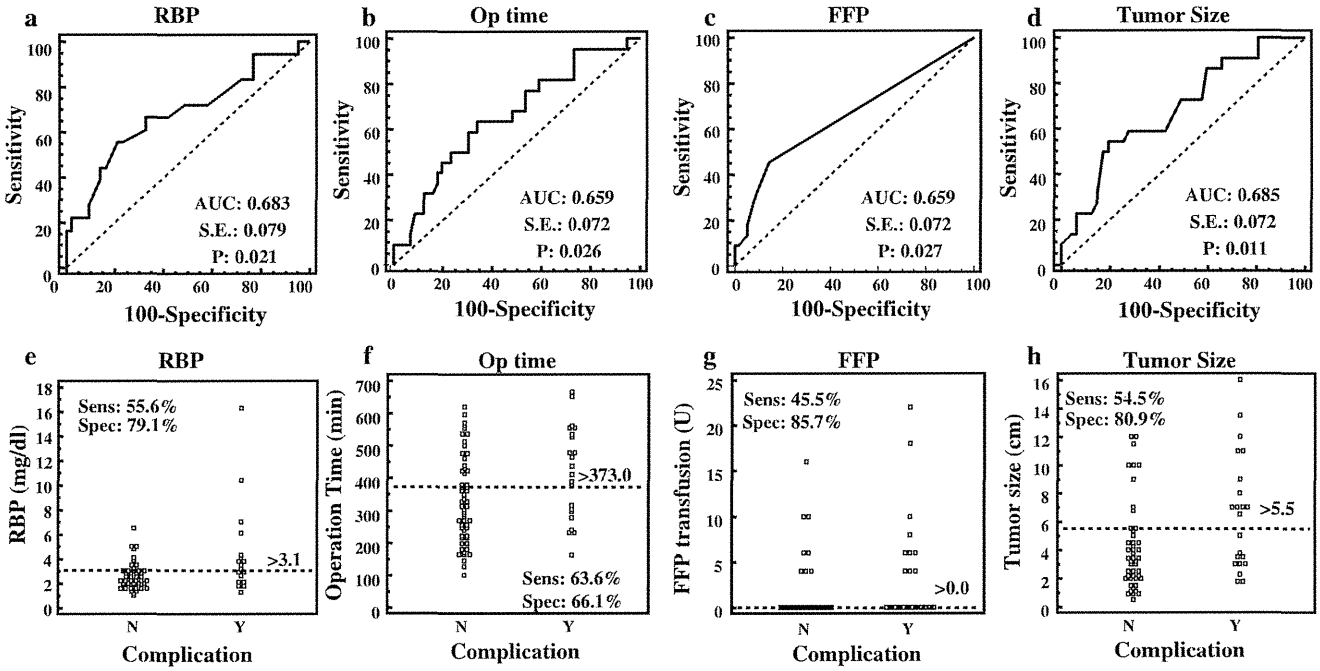

Fig. 1 ROC curves (**a, b, c,** and **d**) and interactive dot diagrams (**e, f, g,** and **h**) of retinol binding protein levels (RBP; **a** and **e**), operative time (Op time; **b** and **f**), fresh frozen plasma transfusion requirement (FFP; **c** and **g**), and tumor size (**d** and **h**). *AUC* area under the ROC curve, *SE* standard error, *CI* confidence interval, *Sens.* sensitivity, *Spec.* specificity, *N* no, *Y* yes. $P < 0.05$ was considered to be significant

determining the optimal surgical management strategy for HCC or predicting the prognosis of HCC patients.

The risk factors for surgical complications in HCC patients can be divided into technical and host factors [13, 14, 18–21]. We did not detect any difference in the frequency of surgical complications between the non-cirrhotic and cirrhotic patients, even though they displayed different risk factors. The extent of liver resection tended to be greater in the noncirrhotic patients, which might have increased their risk of surgical complications. On the other hand, only limited liver resection can be performed in cirrhotic patients due to their poor liver function. Although cirrhotic patients are expected to display a higher risk of surgical complications than noncirrhotic patients due to their immunocompromised condition [18, 21], the limited resections performed in cirrhotic patients might counteract this effect [22]. Another possible reason for the similar complication rates of the two groups is that we might not have performed the operations involving the noncirrhotic patients with sufficient technical skill as the tumors in the complications group were larger than those in the complications-free group, which would have made the procedures more technically difficult. We did not fully elucidate the reason why the noncirrhotic and cirrhotic patients displayed similar complication rates, but we might have to reconsider our surgical management strategy for noncirrhotic patients.

In the noncirrhotic patients, the serum RBP level was found to be a predictive risk factor for surgical complications in addition to tumor size and operative time. Patients' preoperative hepatic reserves are usually evaluated using the Child–Pugh score or liver damage score [3, 23–26]. A previous study found that in noncirrhotic patients these classical liver functional evaluation methods gave similar results for each patient, and it was hard to distinguish between borderline cases [7]. Due to the short half-life of RBP, its serum concentration represents the real-time state of hepatic protein production [23, 27]. In fact, our results suggested that serum RBP levels could be a useful predictor of surgical complications. Therefore, serum RBP levels could be used to predict postoperative complications and determine the hepatic condition of noncirrhotic patients.

Among the noncirrhotic patients, the complications and complications-free groups displayed similar prognoses. The tumors in the complications group were larger than those in the complications-free group, although the two groups displayed similar numbers of tumors. Tumor size and number have been reported to be prognostic factors for HCC patients [3]. However, many of the patients in the complications group had large single tumors. It is possible that tumor size does not have a prognostic impact in cases involving single tumors, but rather, only has a clinical impact in terms of the technical difficulties associated with large tumors. In fact, Truant recently reported that in HCC

Table 3 Clinical demographics of the cirrhotic patients who underwent hepatectomy for hepatocellular carcinoma ($N = 70$)

	Complications	Complications-free	Univariate	Multivariate
Etiology			0.137	
B	9	20		
C	11	22		
BC	1	2		
NBNC	3	2		
Operation			0.339	
0	12	22		
S	4	12		
1	5	9		
2	3	3		
Stage			0.856	
1	6	10		
2	7	18		
3	10	16		
4	1	2		
Operative time (min)	376.7 ± 133.7	319.7 ± 130.2	0.092	
Bleeding (ml)	718.5 ± 677.1	461.9 ± 621.5	0.119	
Blood transfusion (U)	0.83 ± 2.63	0.35 ± 1.31	0.317	
FFP transfusion (U)	3.26 ± 4.33	1.49 ± 4.28	0.115	
Tumor size (cm)	3.28 ± 1.91	2.69 ± 1.46	0.192	
Tumor number	2.4 ± 3.4	1.5 ± 0.9	0.137	
Age (year)	66.5 ± 8.9	65.6 ± 10.3	0.733	
Height (cm)	160.7 ± 8.9	159.4 ± 9.5	0.576	
Weight (kg)	62.4 ± 11.1	60.4 ± 12.2	0.501	
BMI	24.1 ± 3.2	23.7 ± 3.5	0.657	
ALB (g/dl)	3.63 ± 0.39	3.82 ± 0.61	0.174	
Bil (mg/dl)	0.84 ± 0.49	0.74 ± 0.31	0.291	
PT (%)	83.9 ± 10.3	89.9 ± 8.7	0.019	0.007
Plt ($\times 10^4$)	14.2 ± 9.9	13.1 ± 8.3	0.596	
AT (%)	77.5 ± 14.8	82.1 ± 15.9	0.254	
AST (IU/L)	46.8 ± 20.4	47.6 ± 21.5	0.881	
ALT (IU/L)	42.9 ± 25.7	44.2 ± 26.1	0.838	
gGT (IU/L)	94.8 ± 72.5	77.9 ± 84.4	0.428	
CholE (IU/L)	195.3 ± 63.4	236.6 ± 65.7	0.019	0.091
Col (ng/ml)	7.44 ± 2.64	6.53 ± 2.34	0.179	
HA (ng/ml)	270.5 ± 203.3	211.4 ± 157.1	0.189	
BTR	5.22 ± 2.14	5.51 ± 2.32	0.639	
$ICGR_{15}$ (%)	17.4 ± 7.7	12.3 ± 7.3	0.011	0.022
RBP (mg/dl)	2.31 ± 1.22	2.65 ± 1.55	0.392	
PreALB (mg/dl)	14.9 ± 4.9	17.1 ± 7.1	0.194	
HGF (ng/ml)	0.47 ± 0.15	0.42 ± 0.22	0.331	
HH15	0.669 ± 0.101	0.624 ± 0.087	0.069	
LHL15	0.889 ± 0.071	0.911 ± 0.045	0.144	
Child–Pugh score	5.542 ± 0.779	5.222 ± 0.421	0.029	0.182
MELD score	8.595 ± 1.888	7.683 ± 1.374	0.025	0.097

large tumors were associated with a worse prognosis, but some patients whose tumors were not very aggressive achieved better survival regardless of the size of their tumor [28]. Regardless of how tumor size is related to prognosis, surgical complications do not have a prognostic impact in non-cirrhotic patients.

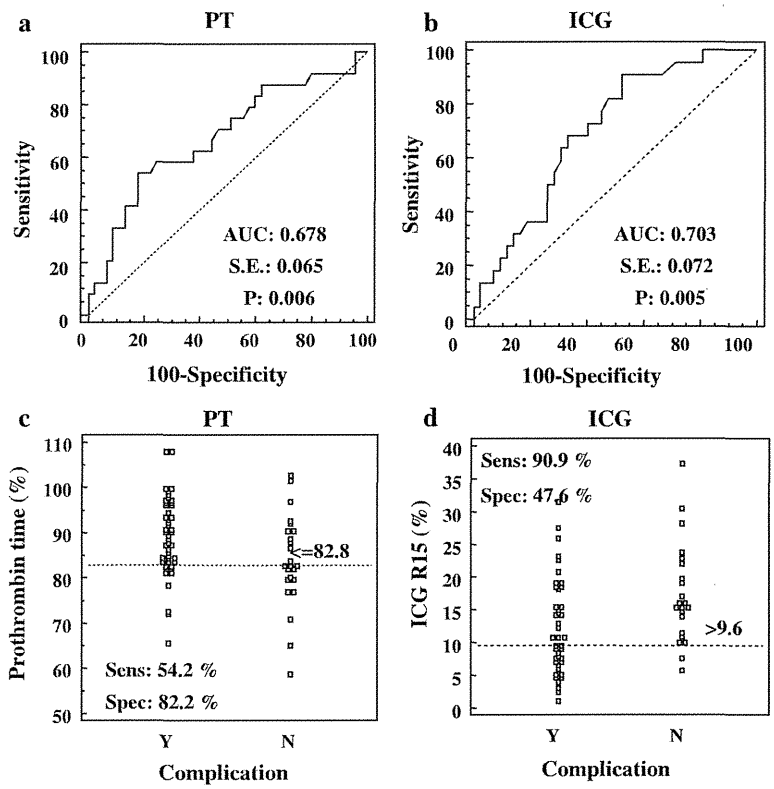

Fig. 2 ROC curves (**a** and **b**) and interactive dot diagrams (**c** and **d**) of prothrombin time (PT; **a** and **c**) and the indocyanine green retention value at 15 min ($ICGR_{15}$; **b** and **d**). *AUC* area under the ROC curve, *SE* standard error, *CI* confidence interval, *Sens.* sensitivity, *Spec.* specificity, *N* no, *Y* yes. $P < 0.05$ was considered to be significant

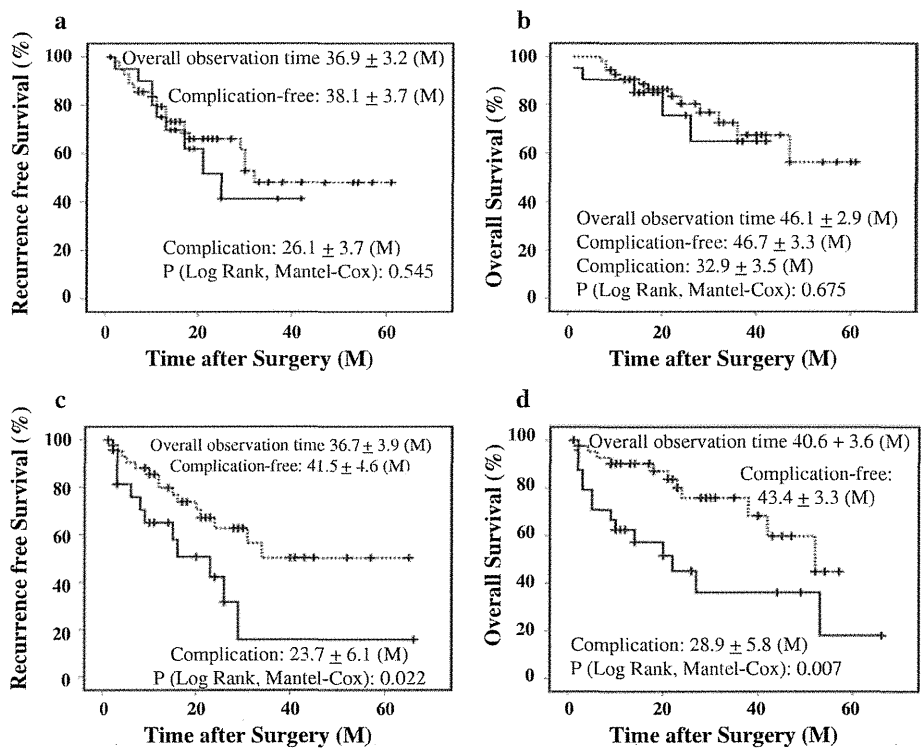

Fig. 3 Recurrence-free survival (**a** and **c**) and overall survival (**b** and **d**) in the noncirrhotic patients (**a** and **b**) and cirrhotic patients (**c** and **d**). *Straight lines* represent the complications group, and *dotted lines* represent the complications-free group

In the cirrhotic patients, PT and the $ICGR_{15}$ were found to be predictors of surgical complications. Although the $ICGR_{15}$ does not count towards the Child–Pugh score, the liver damage score consists of serum albumin and bilirubin levels, PT, and the $ICGR_{15}$ [23, 24]. Thus, our study showed that liver function has a prognostic impact in

cirrhotic patients, as was found in previous reports [14], whereas postoperative complications had no prognostic effect in noncirrhotic patients. Our finding that liver function had a significant impact on postoperative complications in cirrhotic patients has important implications. We found that PT and $ICGR_{15}$ cutoff levels of 80 and 10 %, respectively, can be used to predict which patients will suffer surgical complications and a poor prognosis. Our cutoff values are very similar to those used for the liver damage score. Thus, although the risk of complications is affected by the extent of tumor progression and the type of liver resection in noncirrhotic patients, classical functional evaluations of liver function, such as the Child–Pugh score [17] or liver damage score, are helpful not only for determining surgical indications but also for predicting postoperative complications and prognosis, especially in cirrhotic patients.

On the other hand, the MELD score has been shown to be a predictor of liver failure in cirrhotic patients [29]. However, the MELD score did not achieve statistical significance in the multivariate analysis conducted in the present study. This might have been due to the surgical indications for HCC employed at our institution. Basically, decompensated cirrhotic patients are never considered for liver resection. In addition, there were no patients with hepatorenal syndrome, and most of the patients' serum bilirubin levels were within normal levels. Therefore, the MELD score was dependent on the PT-INR in most patients. Although consecutive studies might be subject to inevitable bias, PT-related scores, including PT itself, might be useful for predicting complications and prognosis in cirrhotic patients.

The morbidity rates of the noncirrhotic patients and cirrhotic patients in our study were not significantly different. The most common complications suffered by the noncirrhotic patients were bile leakage, bleeding, and surgical site infections, including intra-abdominal abscesses and wound infection. On the other hand, the most common complications experienced by the cirrhotic patients were ascites and pleural effusion. The fact that the operations performed in the noncirrhotic patients involved more extensive resections than those performed in the cirrhotic patients, which also affected the resected liver area and wound length, might have caused these responsible these differences. On the other hand, morbidity is inevitable in cirrhotic patients due to their poor systemic condition, which is caused by their poor liver function [7, 14]. The risk of morbidity after liver resection depends on the balance between liver function and operative procedure. Therefore, we need to pay more attention to the surgical management of noncirrhotic patients and the surgical indications and operative plans for cirrhotic patients.

Conclusions

We investigated the predictors of surgical complications after liver resection for HCC according to the pathological background of the patient's liver. In noncirrhotic patients, serum RBP level, tumor size, operation time, and FFP transfusion requirement were found to be predictors of surgical complications, although surgical complications had no prognostic impact in this group. On the other hand, PT and the $ICGR_{15}$ were found to be predictors of surgical complications in the cirrhotic patients, and surgical complications conveyed a significant survival disadvantage in this group.

Surgical strategies for HCC should take the patient's pathological background into account.

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Conflict of interest None

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ORIGINAL ARTICLE

A phase II study of neoadjuvant combination chemotherapy with docetaxel, cisplatin, and S-1 for locally advanced resectable gastric cancer: nucleotide excision repair (NER) as potential chemoresistance marker

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Abstract
Purpose The combination of docetaxel, cisplatin, and S-1 (DCS) chemotherapy is expected to be a promising regimen for advanced gastric cancer. This study was performed to evaluate the efficacy and safety of neoadjuvant DCS chemotherapy for locally advanced resectable gastric cancer.
Methods Patients with locally advanced gastric cancer received 2 courses of preoperative chemotherapy with S-1 (40 mg/m^2 b.i.d.) on days 1–14 and docetaxel (60 mg/m^2) plus cisplatin (60 mg/m^2) on day 8 every 3 weeks, followed by standard curative surgery within 4–8 weeks. The primary endpoint was R0 resectability. Expression of damage DNA binding protein complex subunit 2 (DDB2)/excision repair cross-complementing 1 (ERCC1) in the pretreated tumor tissues was examined by immunohistochemistry.
Results A total of 43 patients received neoadjuvant chemotherapy. The response rate was 74.4 %, and disease control ratio was 100 %. Grade 4 neutropenia developed in 53.5 % of patients and febrile neutropenia in 16.3 %. Non-hematological grade 3/4 adverse events were anorexia (23.3 %), nausea (14.0 %), and diarrhea (23.3 %), but these were generally transient and manageable. The proportion of R0 resections in the 43 eligible patients was 90.7 %, and a pathological response was found in 65.9 % of patients. There were no treatment-related deaths and no major surgical complications. The accuracy of the

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combination of DDB2 and ERCC1 expression for predicting chemoresistance was 82.5 %.
Conclusions Preoperative treatment with DCS combination for locally advanced gastric cancer demonstrated a sufficient R0 resection rate and a good pathological response with manageable toxicities. The DDB2/ERCC1-high phenotype, as determined by immunohistochemistry, may be useful predictor of resistance to DCS chemotherapy.

Keywords Neoadjuvant chemotherapy · Advanced resectable gastric cancer · DSC · Nucleotide excision repair

Introduction

Although the incidence of gastric cancer is decreasing, it remains the second leading cause of cancer-related death globally and in Japan [1]. A further decrease in mortality would require improved treatment outcomes in patients with advanced gastric cancer. Currently, surgery remains the mainstay of curative treatment. However, only an R0 resection is associated with significant cure rates, and less than half of patients with locally advanced gastric cancer will achieve an R0 resection even with aggressive surgery [2]. Despite curative resection, a large proportion of patients with locally advanced gastric cancer will experience recurrence, and the long-term survival rate remains unsatisfactory [3]. The high risk of relapse after surgery has led to the search for strategies to prevent relapse and to improve survival for gastric cancer patients, such as adjuvant therapy or neo-adjuvant approaches.

Recently, the large-scale Japanese phase III trial by the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer group reported the superiority of S-1 as an adjuvant chemotherapy over surgery alone after D2 lymph node dissection of stage II/III patients [4]. Nonetheless, even with adjuvant S-1 chemotherapy, about one-third of R0 patients died within 5 years of surgery, indicating that improved therapeutic strategies are needed.

Preoperative chemotherapy has some theoretical benefits in comparison with postoperative chemotherapy in such patients, including downstaging that increases the possibility of subsequent R0 resection, treating micrometastatic disease early in the course of therapy, evaluating susceptibility to chemotherapy, and generally better tolerability of more intensive chemotherapy. This approach is supported by a large randomized study involving 503 resectable patients; that is, the Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial, the first positive neoadjuvant study, in which the effects of 3 pre- and post-operative cycles of ECF (epirubisine/cisplatin/5-FU) chemotherapy were compared with surgery alone [5]. The study concluded that perioperative chemotherapy decreased the tumor stage and improved patient survival. A similar benefit for perioperative chemotherapy was noted in a French multicenter trial in which 224 patients with potentially resectable gastric cancers were randomly assigned to receive 2–3 cycles of preoperative chemotherapy (CF, 5-FU/cisplatin) or surgery alone [6]. However, 5-year survival rates remain less than 40 % in these trials. Therefore, the development of more effective chemotherapeutic regimens would be required for further improvements of efficacy in neoadjuvant therapy.

During the last decade, several new agents with promising activity against gastric cancer have been identified. These include S-1, docetaxel, and irinotecan [7]. The therapeutic value of combination regimens including these new anticancer agents has been studied with the goal of improving overall treatment efficacy. A phase III study (V325) evaluating the impact of adding docetaxel to CF (DCF) in advanced gastric cancer showed that DCF led to significantly improved outcomes [8]. S-1 is a novel oral fluoropyrimidine, and a recent phase III trial showed that the substitution of S-1 for infusional 5-FU in the CF regimen is comparable in efficacy to 5-FU combined with cisplatin but has significant safety advantages [9]. At present, S-1 plus cisplatin (CS) is recognized as a standard treatment for unresectable advanced or recurrent gastric cancer in Japan [10].

We have previously conducted phase I and phase II studies to evaluate the effect of adding docetaxel to base treatment with S-1 plus cisplatin (DCS) to further improve the therapeutic response; both a very high response rate (87.1 %) and a promising median survival time (687 days) in patients with unresectable advanced gastric cancer were noted [11, 12]. Another phase II study of DCS with a different treatment regimen from ours has been performed by Koizumi et al. [13]; treatment was highly effective (response rate, 81 %), consistent with the results of our study. We also found an appreciable rate of downstaging (25 %) with a very high response rate and no cases of disease progression with this regimen [12], suggesting the applicability of DCS for neoadjuvant chemotherapy. Based on these encouraging results, we performed this multicenter single-arm phase II trial to evaluate the efficacy and safety of preoperative chemotherapy with DCS for locally advanced gastric cancer.

Patients and methods

Patient eligibility

Patients with locally advanced gastric cancer were eligible for the present study. Eligibility criteria included the following: age between 20 and 80 years; PS of 0-1 on the Eastern Cooperative Oncology Group (ECOG) scale;

histologically proven gastric adenocarcinoma; T3-4, N0-3, (or T2N1-3 in the case of diffuse invasive type; linitis plactica), M0 (according to the Japanese Classification of Gastric Carcinoma 13th edition) [14]; clinically diagnosed with potentially resectable tumors; no prior gastric surgery; no previous chemotherapy or radiotherapy; measurable lesion(s) or evaluable disease; no uncontrolled infectious or cardiac disease; adequate renal function; no synchronous or metachronous (within 5 years) malignancy other than carcinoma in situ; and provision of written informed consent. This study was approved by the ethics committee of each institution and hospital.

Baseline evaluation

The pre-study evaluation included physical examination, hematology, biochemistry, urinalysis, chest X-ray, and gastroduodenofiberoscopy. Gastric adenocarcinomas were staged by computed tomography (CT) scan and endoscopic ultrasound (EUS) in order to estimate primary tumor and lymph node status. *Staging* laparoscopy was performed to exclude occult M1 disease in the peritoneum or other intra-abdominal sites. Further examination using radionuclide bone scan, and/or co-registered (18F)-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET)/CT scan was performed if clinically indicated to exclude M1 disease.

Treatment schedule

In this multicenter, nonrandomized, open-label phase II trial, S-1 was administered orally twice daily on days 1–14 at a dose calculated according to the patient's body surface area as follows: <1.25 m^2, 40 mg; 1.25–1.5 m^2, 50 mg; and >1.5 m^2, 60 mg.

Cisplatin was administered by intravenous infusion for 2 h at 60 mg/m^2 in 5 % glucose followed by docetaxel at 60 mg/m^2 in 5 % glucose on day 8 with adequate hydration. Cycles were repeated every 3 weeks. Prophylactic administration of antiemetic medication at a standard dose was routinely used to prevent nausea and vomiting when cisplatin was administered. In the event of toxicity, the treatment delays and dose reductions were planned as previously described [12]. All patients received 2 courses of treatment, and responders received a maximum of 4 courses, followed by standard curative surgery involving a radical resection, the extent of which (total or subtotal gastrectomy) depended on the site of the primary tumor, and D2 or D3 lymphadenectomy within 4–8 weeks.

Assessment and follow-up

Toxicity was evaluated according to the Common Toxicity Criteria for Adverse Events (version 3.0). Assessment of response to neoadjuvant therapy was performed after each preoperative cycle according to Response Evaluation Criteria in Solid Tumors guidelines (version 1.0) and for primary lesions according to the guidelines of the Japanese classification of gastric carcinoma [15]. The pathological response to chemotherapy was classified according to the following criteria provided by the Japanese Gastric Cancer Association (JGCA) [16]: grade 0, no part of tumor affected; grade 1a, less than one-third affected; grade 1b, between one-third and two-thirds affected; grade 2, between two-thirds and entire tumor affected; and grade 3, no residual tumor. A pathological response was defined as one-third or more of the tumor affected (grade 1b, 2 or 3). Each patient was assessed at 1, 3, 6, 9, and 12 months, then every 6 months for 5 years, and then annually or until death.

Immunohistochemistry for ERCC1 and DDB2

Paraffin-embedded tissue sections of gastric cancer tissue were deparaffinized in xylene and treated for 20 min with 0.6 % H_2O_2 to block endogenous peroxidase activity. They were incubated overnight at 4 °C in a 1:100 dilution of mouse monoclonal antibody against ERCC1 (sc-56386, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-DDB2 antibody (ab77765, Abcam, Cambridge, UK). Binding of the primary antibody was detected by peroxidase staining with an avidin–biotin complex system (Dako, Carpinteria, CA, USA). Staining was graded for intensity of staining (1, weak; 2, moderate; 3, strong) and percentage of cells stained (1, 0 to <10 %; 2, 10 to <50 %; 3, 50–100 %). We classified ERCC1 and DBB2 staining as positive when tumor cells showed nuclei reactivity and both scores were two or above, as described previously [17].

Statistical methods

The primary endpoint was the R0 resection rate. The secondary endpoints were pathological response rate, response to chemotherapy, progression-free (PFS) and overall survival (OS), and chemotherapy-related toxicity. Given that the expected rate of R0 resection is 85 % and the threshold incidence is 65 %, based on previously reported data for R0 resection rates in this population [18–20], with an alpha value of 0.025 (1-sided) and a beta value of 0.2, the required number of patients was determined to be 36. The target number of patients was therefore set at 40, accounting for expected dropouts and excluded patients. PFS was defined as the time from registration until objective tumor progression or death. OS was defined as the time from registration until death from any cause. The Fisher's exact probability test was employed for

determining the statistical significance of correlations between marker expression and histological chemotherapeutic effects. P values <0.05 were considered statistically significant.

Results

Patients

From January 2007 to September 2011, 45 patients with locally advanced gastric cancer were enrolled in the study. Two patients did not start chemotherapy for the following reasons: reassessment as inoperable ($n = 1$), and patient request ($n = 1$). Thus, 43 patients were eligible and received chemotherapy. A flow diagram from chemotherapy to surgery is shown in Fig. 1. Patient characteristics are summarized in Table 1. The subjects included 32 men and 11 women, with a median age of 65 years (range 31–78 years). Most of these patients were in good general condition (74.4 % with a performance status of 0). Histologically, 17 (39.5 %) patients had well-differentiated adenocarcinomas and 26 (60.5 %) had undifferentiated adenocarcinomas. On baseline EUS and CT, 7 patients (16.3 %) had T4 tumors and 39 patients (90.7 %) had N+ disease including N2 bulky mass (6.9 %, 3/43) or N3 paraaortic nodes metastases (9.3 %, 4/43).

Preoperative chemotherapy

Forty-three patients were administered a total of 108 courses, with a median of 2 courses (range 1–4). While all patients received course 1, 2 of them did not receive course 2 of preoperative chemotherapy but underwent surgery

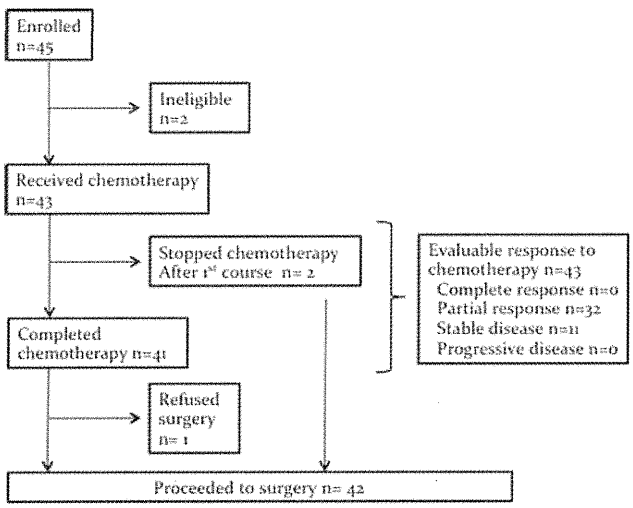

Fig. 1 Trial profile

Table 1 Patient characteristics at baseline ($N = 43$)

Characteristics	No. of patients	%
Age, years		
Median	65	
Range	31–78	
Sex		
Male	32	74.4
Female	11	25.6
Performance status		
0	32	74.4
1	11	25.6
Histology		
Intestinal type	17	39.5
Diffuse type	26	60.5
T stage (JGCA)		
T2	7	16.3
T3	29	67.4
T4	7	16.3
N stage (JGCA)		
N0	4	9.3
N1	8	18.6
N2	27	62.8
N3	4	9.3
Stage		
II	2	4.7
IIIA	15	34.9
IIIB	20	46.5
IV	6	13.9

(Fig. 1), due to physician's impression of poor tolerance and patient refusal (1 patient each). Thus, the completion rate of 2 courses was 95.3 % (41/43), and in the second course, 90 % delivery of the planned dose was achieved for S-1, docetaxel, and CDDP. Among patients who responded to treatment and were deemed by their physician after course 2 to be able to tolerate subsequent courses, 18 patients received a third course and 6 patients received a fourth course. A treatment delay of 7 or more days was noted in 13 of the courses. The clinical response rate (complete response + partial response) was 74.4 % (95 % CI, 61.4–87.4 %), and no patient had disease progression during pre-operative chemotherapy period. The incidence of hematological grade 4 adverse events was as follows: leukocytopenia, 37.2 %; neutropenia, 53.5 %; anemia, 2.3 %; febrile neutropenia, 2.3 %. Non-hematological grade 3 or higher adverse events were anorexia, 23.3 %; nausea, 14.0 %; vomiting, 7.0 %; and diarrhea, 23.3 % (Table 2). There were no chemotherapy-related deaths. All treatment-related toxicities resolved with appropriate care, and no treatment-related deaths were observed.

Table 2 Adverse events occurring during chemotherapy

Toxicity (NCI–CTC)	No. of patients (%)			
	Grade			
	1	2	3	4
Hematologic				
Leucopenia	1 (2.3)	1 (2.3)	18 (41.9)	16 (37.2)
Neutropenia	1 (2.3)	1 (2.3)	13 (30.2)	23 (53.5)
Anemia	4 (9.3)	8 (18.6)	2 (4.7)	1 (2.3)
Febrile neutropenia	–	–	6 (14.0)	1 (2.3)
Thrombocytopenia	5 (11.6)	4 (9.3)	1 (2.3)	0 (0.0)
Nonhemotological				
Anorexia	4 (9.3)	15 (34.9)	10 (23.3)	0 (0.0)
Nausea	5 (11.6)	9 (20.9)	6 (14.0)	0 (0.0)
Vomiting	4 (9.3)	3 (7.0)	3 (7.0)	0 (0.0)
Diarrhea	4 (9.3)	1 (2.3)	10 (23.3)	0 (0.0)
Stomatitis	6 (14.0)	4 (9.3)	1 (2.3)	0 (0.0)
Fatigue	8 (18.6)	6 (14.0)	0 (0.0)	0 (0.0)
AST/ALT elevation	3 (7.0)	3 (7.0)	0 (0.0)	0 (0.0)
Creatinine elevation	3 (7.0)	3 (7.0)	0 (0.0)	0 (0.0)

Surgical findings and surgical pathology

A total of 42 patients proceeded to surgery (Fig. 1; Table 3). Resection with curative intent was undertaken in only 41 patients because 1 patient underwent only gastrojejunostomy due to *localized peritoneal* metastases and *pancreatic invasion*. Of the 41 patients who had resection with curative intent, R0 resection was performed in 39, R1 in 1 (positive microscopic margin), and R2 in 1 with unresectable peritoneal metastases. Thus, the proportion of R0 resections in the 43 eligible patients was 90.7 % (95 % CI, 82.0–99.4 %). Among the 41 resected patients, 25 had D2 lymph node dissection, and the remaining 16 had D3 lymph node dissection. Postoperative complications were observed in 9 patients (21.4 %). The actual complications were as follows: delayed gastric emptying, wound infection, deep vein thrombosis, abdominal abscess, abdominal fluid collection, and ileus. Overall, there was no mortality and there were no serious complications. Of the 41 operated patients for whom data regarding surgical pathologic staging were available, 25 patients (61.0 %) had a decrease of at least 1 level in their T stage and 6 patients (15.4 %) with N+ disease had post-treatment N0 disease. Overall, gastric tumors were down staged in 28 patients (68.3 %), unchanged in 9 patients (21.9 %), and upstaged in 4 patients (9.8 %).

Survival analysis

The median follow-up time was 30.8 months. At the time of the analyses (April 1, 2012), 41 patients (95.3 %) were still alive; The median PFS and MST were not reached, the 3-year PFS was 85.9 % (95 % CI, 75.5–96.3), and the 3-year OS was 89.7 % (95 % CI, 80.6–98.8) (Fig. 2).

Table 3 Surgical and pathologic results

	No. of patients	%
Surgery results		
All	42	100
Types of surgery		
Total gastrectomy	34	80.9
Distal gastrectomy	7	16.7
Bypass surgery	1	2.4
Lymph node dissection		
No dissection	1	2.4
D2	25	59.5
D3	16	38.1
Extent of resection		
No resection	1	2.4
R0	39	92.8
R1	1	2.4
R2	1	2.4
Pathology results		
All	41	100
T stage (JGCA)		
T0	2	4.9
T1	4	9.8
T2	11	26.8
T3	21	51.2
T4	3	7.3
N stage (JGCA)		
N0	10	21.1
N1	12	31.6
N2	14	36.8
N3	5	10.5
M status (JGCA)		
M0	39	94.7
M1	2	5.3

JGCA Japanese Gastric Cancer Association

Chemotherapeutic effects and DDB2/ERCC1 expression in pretreatment biopsy specimens from gastric cancer patients treated with neoadjuvant DCS regimen

Excision repair cross-complementing 1 (ERCC1) is a key enzyme in the nucleotide excision repair (NER) pathway, and its expression is reported to be a useful predictor of the clinical outcome of advanced gastric cancer patients treated with platinum-based chemotherapy [17, 21, 22]. On the other hand, damage DNA binding protein complex subunit 2 (DDB2) was found to serve as the initial damage

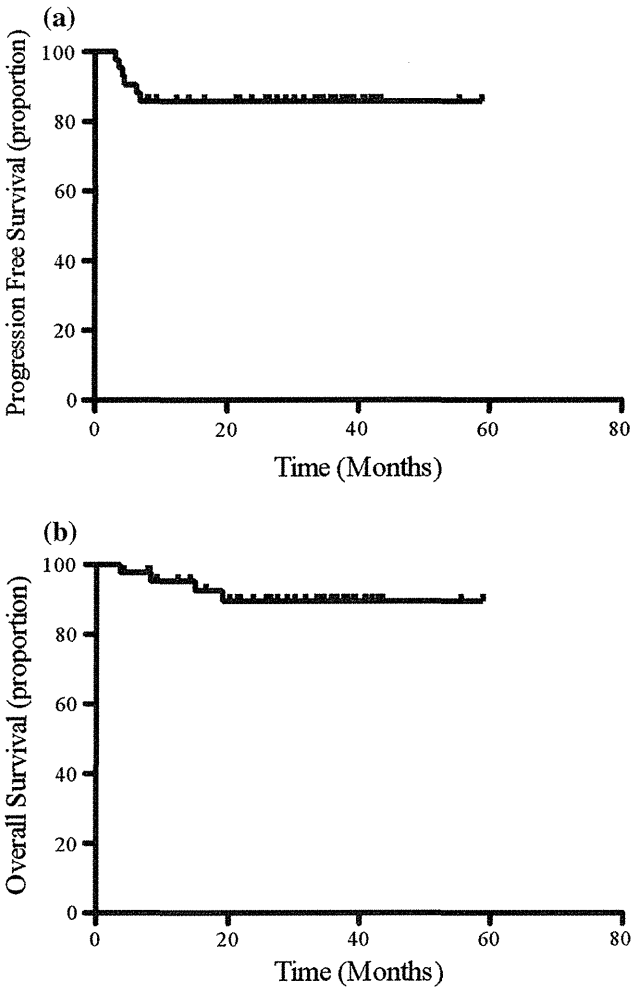

Fig. 2 Kaplan–Meier curves for **a** progression-free survival and **b** overall survival

recognition factor during NER, and we reported that loss of DDB2 repair function contribute to cancer susceptibility and cellular sensitivity to DNA damage [23]. We therefore examined the possible correlation between anti-tumor effect of DCS therapy (pathological response) and the expression of DDB2 and/or ERCC1 in pretreated tumor tissues by immunohistochemical staining. Table 4 shows the relationship between chemotherapeutic effects and marker expression in the pretreatment biopsy specimens. Histological chemotherapeutic responders consisted of 27 (65.9 %) out of 41 resected cases; grade 1b, 10 patients (24.4 %), grade 2, 15 patients (36.6 %); and grade 3, 2 patients (4.9 %). The remaining 14 patients (34.1 %) were categorized as nonresponders; grade 0, 1 patient (2.4 %); and grade 1a, 13 patients (31.7 %).

Adequate biopsy material was available in 40 out of the 43 cases prior to receiving neoadjuvant chemotherapy. High DDB2 expression was observed in 4 lesions (14.8 %) of 27 responders and in 8 lesions (61.5 %) of 13 nonresponders: statistical significance was noted between responders and nonresponders ($P = 0.0065$). The accuracy of DDB2 expression for predicting chemoresistance was 77.5 %; that is, 31 (23 responders and 8 nonresponders) out of 40 patients treated with neoadjuvant chemotherapy.

High expression of ERCC1 was observed in 8 lesions (61.5 %) of the nonresponders and in 5 lesions (18.5 %) of the responders: there was a significant difference between responders and nonresponders ($P = 0.029$). The accuracy of ERCC1 expression for predicting chemoresistance was 75.0 %; that is, 30 (22 responders and 8 nonresponders) out of 40 patients treated with neoadjuvant chemotherapy.

The DDB2- and/or ERCC1-high phenotype was observed in 13 lesions (100 %) of the nonresponders and in 7 lesions (25.9 %) of the responders: The difference between responders and nonresponders was statistically significant ($P < 0.0001$). The accuracy of the combination of DDB2 and ERCC1 expression for predicting chemoresistance was 82.5 %; that is, 33 (20 responders and 13 nonresponders) out of 40 patients treated with neoadjuvant chemotherapy.

Discussion

New chemotherapeutic regimens for advanced gastric cancer including taxanes, oral pyrimidine, and CPT-11

Table 4 Relationship between expression of DDB2 and ERCC1, and effects of neoadjuvant DCS chemotherapy in pretreatment biopsy specimens

Marker expression	Pathological responders (%) $N = 27$[a]	Pathological nonresponders (%) $N = 13$[a]	P value	Accuracy (%)
DDB2				
Positive	4 (14.8)	8 (61.5)	0.0065	77.5
Negative	23 (84)	5 (33)		
ERCC1				
Positive	5 (18.5)	8 (61.5)	0.029	75.0
Negative	22 (81.5)	5 (42)		
DDB2 and/or ERCC1-positive	7 (25.9)	13 (100)	<0.0001	82.5
DDB2 and ERCC1-negative	20 (72)	0 (0)		

[a] Pretreatment biopsy specimens were available for analysis in 40 out of 43 patients with neoadjuvant chemotherapy

have been developed and have proven to be highly effective [7]. Consequently, neoadjuvant chemotherapy using these new drugs is expected to improve the prognosis of advanced gastric cancer. We therefore evaluated the efficacy of a triple regimen including docetaxel, S-1, and CDDP (DCS) employed as neoadjuvant chemotherapy in patients with clinically resectable locally advanced gastric cancer. With this regimen, we achieved a high R0 resection rate, as expected, without an increase of operative morbidity and operative mortality in patients with relatively high-risk backgrounds.

It is generally assumed that low resectability is responsible for the poor prognosis of advanced gastric cancer patients. A number of clinical trials have shown that preoperative chemotherapy is feasible and able to increase the rate of R0 resection [24]. The response rate in previous neoadjuvant chemotherapy trials showed modest to moderate activity (40–60 % response rate) and R0 resection rates up to 83 % [25, 26]. Accordingly, there is a need to improve the response rate to achieve a further increase in R0 resection rates with treatment for advanced gastric cancer.

The high activity of the DCS combination (ORR 74.4 %; 95 % CI, 61.4–87.4 %, disease control rate; 100 %) in this study is in accordance with our previous trial for first-line treatment in unresectable metastatic gastric cancer [11, 12] and compares favorably with other active chemotherapy regimens reported in this setting [19, 25, 26]. This indicates that the DCS regimen may be an effective treatment option in the neoadjuvant setting, where high anti-tumor activity resulting in a high down-staging rate, and no progressive disease cases are required. In fact, downstaging was observed in 68.3 % of patients, and the R0 resection rate achieved in the present study (39/43, 90.7 %; 95 % CI, 82.0–99.4 %) was among the highest R0 rates reported [26]. It may not be justified to simply compare our results with those of other studies, since R0 resection rates are influenced by the patients' backgrounds and the operational definition of unresectability. Our patients' backgrounds were, however, relatively dominated by marginally resectable gastric cancers: para-aortic nodal metastases were seen in 9.3 % and bulky N2 in 6.9 % of the cases. Para-aortic lymph node (JGCA-N3) enlargement is regarded as unresectable distant metastases (M1) in the UICC TNM staging system, and usually patients with JGCA-bulky N2 rarely survive for more than 3 years when treated by chemotherapy alone or by surgery followed by postoperative chemotherapy [19, 27].

In this study, the R0 resection rate was nevertheless as high as 90.7 % (100 % in N3 and 67 % in T4 cases). Therefore, preoperative DCS chemotherapy might strongly promote tumor regression, eradicate nodal or possible peritoneal metastases, and improve resectability in patients with marginally resectable gastric cancer.

This regimen's effectiveness was also indicated by the fact that the pathological response rate was as high as 65.9 %. Although similar criteria for histopathological regression have been used in several studies, these criteria are not standardized and may be investigator dependent. Several studies of neoadjuvant chemotherapy employing the same Japanese criteria that were used in the present study reported pathological response rates of 51 and 48 % for the JCOG0405 [28] and JCOG0210 [29] trials, respectively, using the S-1/CDDP regimen, and 15 % for the JCOG0001 trial using the CDDP/CPT-11 regimen [19]. Hence, DCS neoadjuvant chemotherapy showed a much better therapeutic effect than other CDDP-based regimens.

There is a correlation between increased pathologic response to therapy and survival in retrospective studies [30]. Therefore, our regimen, which induced a high pathological response rate, is expected to bring about a good prognosis. Despite a short follow-up period, the 3-year OS of 89.7 % and 3-year PFS of 85.9 % in this study are also encouraging.

The degree of toxicity of neoadjuvant chemotherapy is a critical problem because of its potential adverse effects on operative morbidity and operative mortality. Like other docetaxel-containing triple regimens in which hematological toxicity was the major adverse effect [8], the DCS regimen was associated with a high incidence of severe neutropenia, which occurred in 53.5 % of patients in the neoadjuvant setting.However, febrile neutropenia occurred in only 16.3 % (grade 4; 2.3 %) of patients; all of these cases were transient and manageable with G-CSF administration and had dose reductions that prevented the recurrence of toxicity. Obviously, DCS treatment necessitates careful observation of these toxicity patterns to prevent treatment-associated toxicities. In fact, in our trial, 95.3 % of patients were able to receive the 2 planned courses of preoperative chemotherapy. Moreover, there was no increase in operative morbidity and no operative mortality as compared with patients who underwent identical surgery for gastric cancer at our institution during the same time period but who did not receive preoperative therapy.

Resistance to chemotherapy would be a serious problem in the successful treatment of gastric cancers especially in a neoadjuvant setting. In particular, for those patients who had achieved little or no response to preoperative chemotherapy, the use of alternative forms of adjuvant therapy could be considered to improve outcomes. Therefore, to identify chemoresistance markers, we focused on key DNA repair and damage signaling factors, since the antitumor activity of platinum-based chemotherapy is largely dependent on the DNA repair capacity of cancer cells. We showed that nuclear expression of ERCC1 is significantly associated with resistance to chemotherapy, consistent with

reports of other CDDP-based regimen such as 5-FU/oxaliplatin [17], ECF/ECX chemotherapy [21], and CDDP/S-1 or irinotecan [22]. In addition, we have provided the first evidence that DDB2, also as important NER factor, protein expression in pretreatment biopsy specimens is predictive of gastric cancer chemosensitivity. Moreover, we have shown that the accuracy for predicting chemoresistance to DCS was 82.5 % when DDB2 expression was combined with ERCC1 expression, whereas the predictive accuracy was only 77.5 % for DDB2 expression and 75.0 % for ERCC1 expression. These results indicate that the DDB2- and/or ERCC1-high phenotype as determined, by immunohistochemistry, is a strong predictor of resistance to DCS chemotherapy.

In summary, the results of the current study indicate that the DCS regimen is feasible and highly effective as neoadjuvant chemotherapy for locally advanced gastric cancer patients. These results warrant further large-scale investigation of the DCS regimen in a neoadjuvant setting especially for the treatment of marginally resectable gastric cancer.

Conflict of interest The authors have no conflict of interest.

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Expression and Function of *FERMT* Genes in Colon Carcinoma Cells

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Abstract. *Invasion into the matrix is one of hallmarks of malignant diseases and is the first step for tumor metastasis. Thus, analysis of the molecular mechanisms of invasion is essential to overcome tumor cell invasion. In the present study, we screened for colon carcinoma-specific genes using a cDNA microarray database of colon carcinoma tissues and normal colon tissues, and we found that fermitin family member-1 (FERMT1) is overexpressed in colon carcinoma cells. FRRMT1, FERMT2 and FERMT3 expression was investigated in colon carcinoma cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that only FERMT1 had cancer cell-specific expression. Protein expression of FERMT1 was confirmed by western blotting and immunohistochemical staining. To address the molecular functions of FERMT genes in colon carcinoma cells, we established FERMT1-, FERMT2- and FERMT3-overexpressing colon carcinoma cells. FERMT1-overexpressing cells exhibited greater invasive ability than did FERMT2- and FERMT3-overexpressing cells. On the other hand, FERMT1-, FERMT2- and FERMT3-overexpressing cells exhibited enhancement of cell growth. Taken together, the results of this study indicate that FERMT1 is expressed specifically in colon carcinoma cells, and has roles in matrix invasion and cell growth. These findings indicate that FERMT1 is a potential molecular target for cancer therapy.*

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Colon carcinoma is a major malignancy, with a high mortality rate. In the process of tumorigenesis, tumor cells undergo multiple steps of genetic events (1), and multiple steps are also required for the cells to obtain several different phenotypes. Tissue invasion and metastasis are hallmarks that distinguish malignant from benign diseases (2). Several classes of proteins are involved in the process of tissue invasion; however, the exact molecular mechanisms of invasion remain unclear.

Fermitin family member (*FERMT*) genes include *FERMT1*, *FERMT2* and *FERMT3*, and these genes have been reported to be mammalian homologs of the *Caenorhabditis elegans* gene (3,4). The *unc-112* gene mutant had a phenotype similar to that of *unc-52* (perlecan), *pat-2* (α-integrin) and *pat-3* (β-integrin) mutants, and *unc-112* has been described as a novel matrix-associated protein (3). In subsequent studies, *FERMT2* was found to be related to invasion in MCF-7 breast carcinoma cells (5). *FERMT1* has been reported to be overexpressed in lung carcinoma cells and colon carcinoma cells (4), and has been reported to be related to invasion of breast carcinoma cells (6). However, the molecular functions of *FERMT1* in colon carcinoma cells remain elusive.

In this study, we screened a gene expression database of carcinoma tissues to analyze the molecular mechanisms of colon carcinoma, and we isolated *FERMT1* as a gene overexpressed in colon carcinoma tissues. We then analyzed the molecular functions of *FERMT* genes in colon carcinoma cells.

Materials and Methods

Cell lines, culture, cell growth assay and gene transfer. Colon adenocarcinoma cell lines HCT116, HCT15, Colo205, SW480, CaCO2, RTK, SW48, LoVo, DLD1, HT29 and Colo320 were kind gifts from Dr. K. Imai (Sapporo, Japan), and the KM12LM cell line was a kind gift from Dr. K. Itoh (Kurume, Japan). All cell lines were

cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Japan, Tokyo, Japan).

For cell growth assay, 1×10^5 cells were seeded in a 6-well plate, and total cell numbers were counted every day by using Countess™ (Life Technologies).

A retrovirus system was used for gene transfer, as described previously (7). Briefly, a pMXs-puro retroviral vector was transfected into PLAT-A amphotropic packaging cells (kind gift from Dr. T. Kitamura), and then HCT116 and SW480 cells were infected with the retrovirus. Puromycin was added at 5 μg/ml for establishment of stable transformants.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of FERMT genes in normal tissues and colon carcinoma cells. RT-PCR analysis was performed as described previously (8). Primer pairs used for RT-PCR analysis were 5'-GTCTGCTGAAACACAGGATTT-3' and 5'-GTTTTTCTAGTGGTTCTCCTT-3' for *FERMT1*, with an expected PCR product size of 272 base pairs (bps); 5'-CATGACATCAGAGAATCATTT-3' and 5'-ACTGGATTCTTCTTT GCTCTT-3' for *FERMT2*, with an expected PCR product size of 256 bps; 5'-AAAGTTCAAGGCCAAGCAGCT-3' and 5'-TGAAGGCCA CATTGATGTGTT-3' for *FERMT3* with an expected PCR product size of 326 bps; and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with an expected product size of 452 bps. *GAPDH* was used as an internal control. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.2% agarose gel. Nucleotide sequences of the PCR products were confirmed by direct sequencing.

Construction of plasmids and transfection. Full-length *FERMT1*, *FRERMT2* and *FERMT3* cDNAs were amplified from cDNA of LoVo cells with PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The primer pairs were 5'-CGGGGTACCATGCTGTCATCC ACTGACTTT-3' as a forward primer and 5'-CCGCTCGAGATCCTG ACCGCCGGTCAATTT-3' as a reverse primer (underlines indicating *KpnI* and *XhoI* recognition sites, respectively) for *FERMT1*, 5'-CGGGGTACCGCCACCATGGCTCTGGACGGGATAAGG-3' as a forward primer and 5'-CCGCTCGAGCACCCAACCACTGGTA AGTTT-3' as a reverse primer for *FERMT2*, and 5'-CGGGGTACC GCCACCATGGCGGGGATGAAGACAGCC-3' as a forward primer and 5'-CCGCTCGAGGAAGGCCTCATGGCCCCCGGT-3' as a reverse primer for *FERMT3*. The PCR product was inserted into the pcDNA3.1 expression vector (Life Technologies) fused with a FLAG-tag. The cDNA sequences were confirmed by direct sequencing, and proved to be identical as reported previously (4). The inserts were then sub-cloned into a pMXs-puro retrovirus vector (kind gift from Dr. T. Kitamura, Tokyo, Japan). For the construct of protein expression, a *BglII* and *XhoI*-digested deletion mutant of *FERMT1* cDNA that was amplified by PCR using the primer pair 5'-GAAGATCTATGCT GTCATCCACTGACTTT-3' and 5'-CCGCTCGAGATCCTGACCGC CGGTCAATTT-3' (underlines indicating *BglII* and *XhoI* recognition sites, respectively) was inserted into a *BamHI* and *XhoI*-digested pQE30 (Qiagen Japan, Tokyo, Japan) vector.

FERMT1 recombinant protein production and establishment of a monoclonal antibody (mAb). A pQE30-*FERMT1* deletion mutant construct was transformed into Escherichia coli strain M15 (Qiagane Japan, Tokyo, Japan), and His6 tag-fused FERMT1 protein was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30°C. Cells were lysed in lysis buffer [6 M guanidine hydrochloride, 20 mM HEPES (pH 8.0), 50 mM NaCl], and recombinant FERMT1 protein was purified using Ni-NTA resin (Qiagen Japan).

The FERMT1 recombinant protein (100 μg) was used for immunization of BALB/c mice (CHARLES RIVER LABORATORIES JAPAN, INC., Yokohama, Japan) by intraperitoneal (*i.p.*) injection four times at two-week intervals. One week after the last injection, splenic cells were collected and fused with the NS-1 mouse myeloma cell line (ATCC, Manassas, VA, USA) at a 4:1 ratio. FERMT1 protein-specific hybrydomas were screened with enzyme-linked immunosorbent assay (ELISA) and western blotting using recombinant FERMT1 protein.

Immunohistochemical staining and western blotting. Immunohistochemical staining was performed with a colon carcinoma tissue microarray established from formalin-fixed surgically-resected tumor specimens of colon carcinoma at Sapporo the Medical University Hospital, as described previously (8). Anti-FERMT1 antibody was used at a 10-fold dilution with the anti-FERMT1-specific hybridoma culture supernatant. Western blotting of colon carcinoma tissues and colon carcinoma cells was performed as described previously (8). Anti-FERMT1 antibody was used at a 10-fold dilution with hybridoma culture supernatant.

Matrigel invasion assay. BD BioCoat Matrigel Invasion Chambers (Discovery Labware, Bedford, MA, USA) and polyethylene terephthalate (PET) track-etched membranes with pore sizes of 8.0 μm (Becton Dickinson, San Diego, CA, USA) were used for the invasion assay, according to the protocol of the manufacturer. HCT116- and SW480-transformant cells (2.5×10^4 cells/500 ml) were plated in the top chamber in DMEM, and culture medium with 10% FBS was used in the bottom chamber as a chemoattractant. Twenty-four hours later, cells were fixed and stained using a HEMA 3 STAT Pack (Fisher Scientific Japan, Tokyo, Japan). Cell numbers were counted on microphotographs taken in ten areas of the membrane.

Statistical analysis. In cell growth assays and invasion assays, samples were analyzed using Student's *t*-test, with $p<0.05$ conferring statistical significance.

Results

Isolation of the colon carcinoma-related gene FERMT1. We screened a gene expression database of approximately 700 normal organ tissues and about 4000 carcinoma tissues using the Affymetrix GeneChip Human Genome U133 Array Set that contains approximately 39,000 genes. One of the genes that was overexpressed in colon carcinoma tissues was shown to be *FERMT1*, a member of the *FERMT* gene family. In a previous study, *FERMT1* was shown to be overexpressed in lung carcinoma cells and colon carcinoma cells (4). FERMT1 is member of a family of highly homologous gene products including FERMT2 and FERMT3 (Figure 1A). *FERMT1*, *FERMT2* and *FERMT3* share a FERM domain and a Pleckstrin homology domain (PH) domain, which are a cytoskeletal-associated domain and phosphatidylinositol