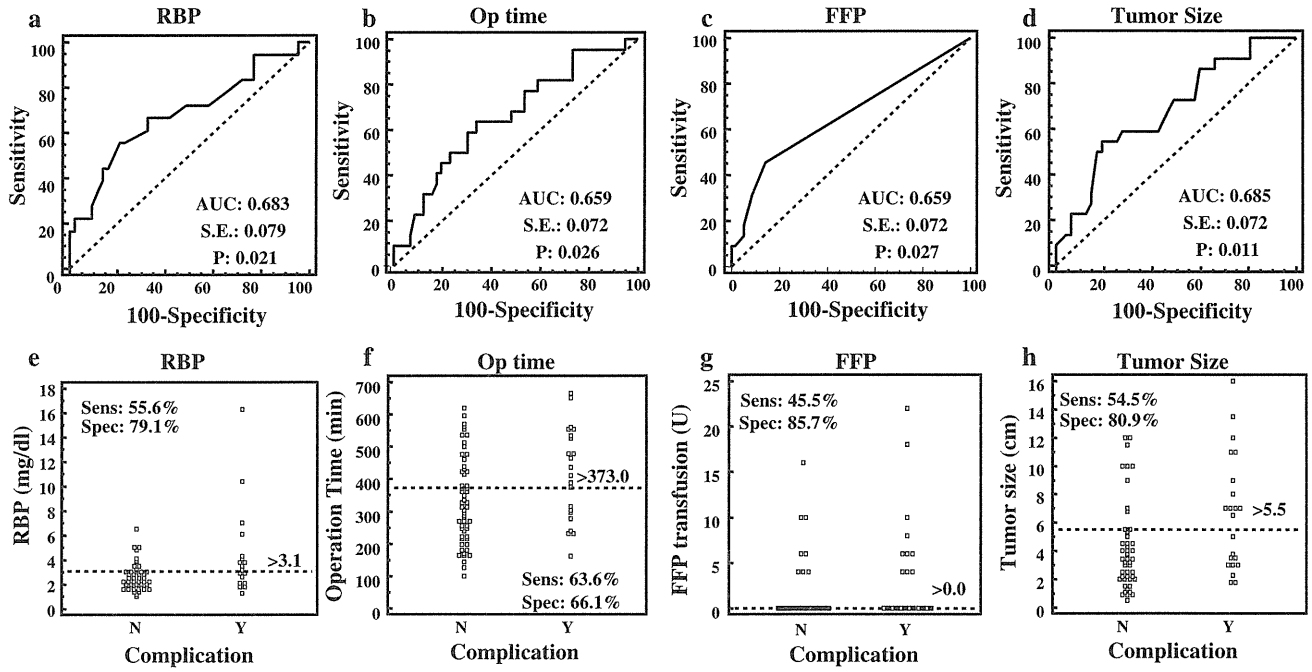

Fig. 1 ROC curves (**a, b, c,** and **d**) and interactive dot diagrams (**e, f, g,** and **h**) of retinol binding protein levels (RBP; **a** and **e**), operative time (Op time; **b** and **f**), fresh frozen plasma transfusion requirement (FFP; **c** and **g**), and tumor size (**d** and **h**). *AUC* 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.

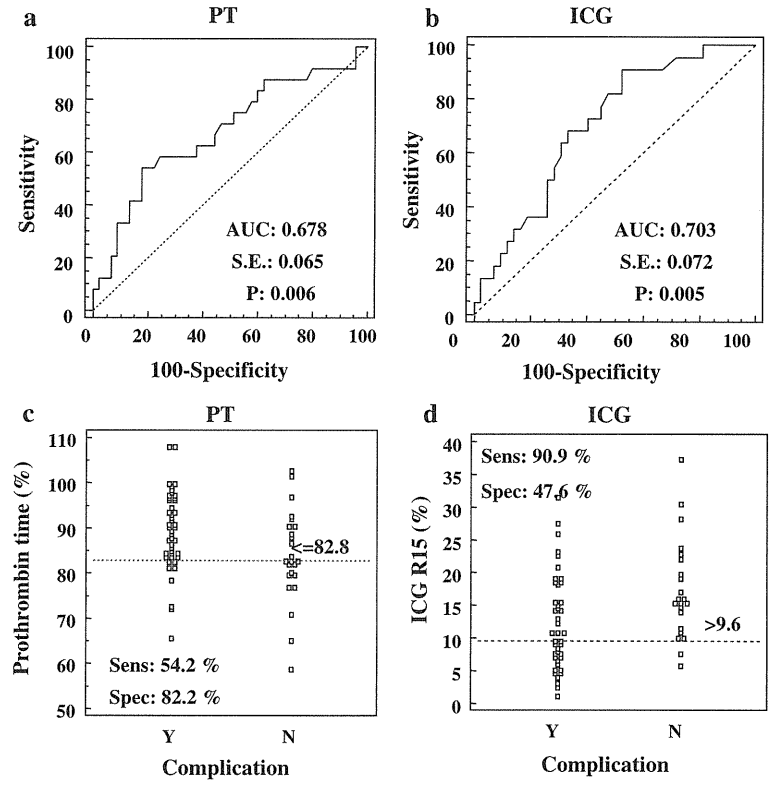

Fig. 2 ROC curves (**a** and **b**) and interactive dot diagrams (**c** and **d**) of prothrombin time (PT; **a** and **c**) and the indocyanine green retention value at 15 min ($ICGR_{15}$; **b** and **d**). *AUC* area under the ROC curve, *SE* standard error, *CI* confidence interval, *Sens.* sensitivity,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

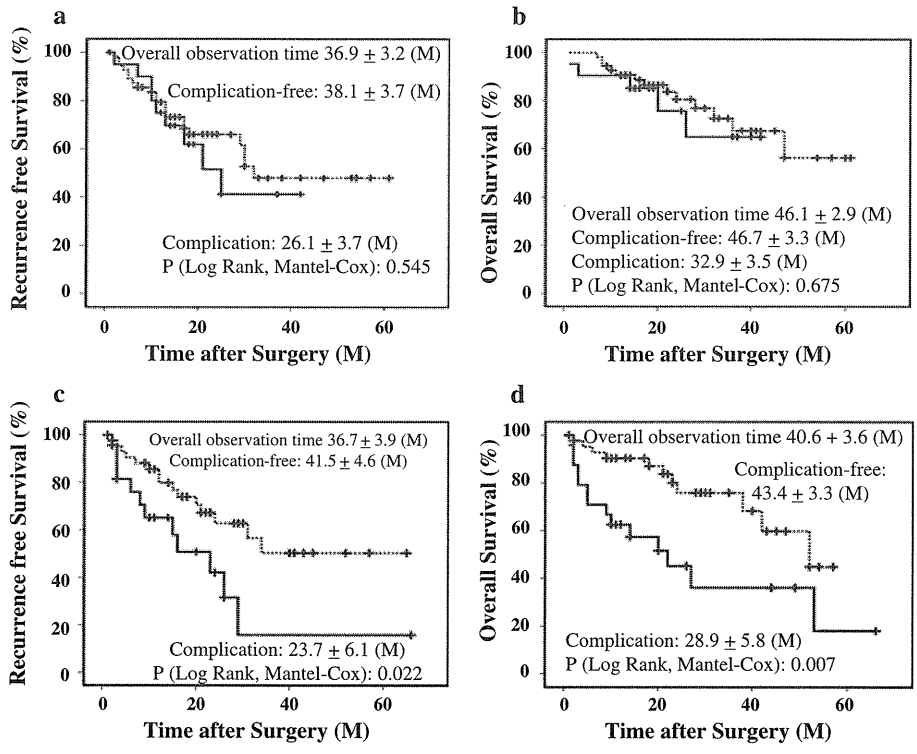

Fig. 3 Recurrence-free survival (**a** and **c**) and overall survival (**b** and **d**) in the noncirrhotic patients (**a** and **b**) and cirrhotic patients (**c** and **d**). *Straight lines* represent the complications group, and *dotted lines* represent the complications-free group

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

References

1. Forner A, Llovet JM, Bruix J (2012) Hepatocellular carcinoma. Lancet 379:1245–1255
2. El-Serag HB (2012) Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology 142:1264–1273
3. Vauthey JN, Dixon E, Abdalla EK et al (2010) Pretreatment assessment of hepatocellular carcinoma: expert consensus statement. HPB (Oxford) 12:289–299
4. Chow PK (2012) Resection for hepatocellular carcinoma: is it justifiable to restrict this to the American Association for the Study of the Liver/Barcelona Clinic for Liver Cancer criteria? J Gastroenterol Hepatol 27:452–457
5. Starley BQ, Calcagno CJ, Harrison SA (2010) Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology 51:1820–1832
6. Reddy SK, Steel JL, Chen HW et al (2012) Outcomes of curative treatment for hepatocellular cancer in nonalcoholic steatohepatitis versus hepatitis C and alcoholic liver disease. Hepatology 55:1809–1819
7. Mizuguchi T, Katsuramaki T, Nobuoka T et al (2004) Serum hyaluronate level for predicting subclinical liver dysfunction after hepatectomy. World J Surg 28:971–976. doi:10.1007/s00268-004-7389-1
8. Takayama T (2011) Surgical treatment for hepatocellular carcinoma. Jpn J Clin Oncol 41:447–454
9. Rahbari NN, Mehrabi A, Mollberg NM et al (2011) Hepatocellular carcinoma: current management and perspectives for the future. Ann Surg 253:453–469
10. Bryant R, Laurent A, Tayar C et al (2009) Laparoscopic liver resection-understanding its role in current practice: the Henri Mondor Hospital experience. Ann Surg 250:103–111
11. Reddy SK, Tsung A, Geller DA (2011) Laparoscopic liver resection. World J Surg 35:1478–1486. doi:10.1007/s00268-010-0906-5

12. Koniaris LG, Levi DM, Pedroso FE et al (2011) Is surgical resection superior to transplantation in the treatment of hepatocellular carcinoma? Ann Surg 254:527–537
13. Chok KS, Ng KK, Poon RT et al (2009) Impact of postoperative complications on long-term outcome of curative resection for hepatocellular carcinoma. Br J Surg 96:81–87
14. Mizuguchi T, Nagayama M, Meguro M et al (2009) Prognostic impact of surgical complications and preoperative serum hepatocyte growth factor in hepatocellular carcinoma patients after initial hepatectomy. J Gastrointest Surg 13:325–333
15. Dindo D, Demartines N, Clavien PA (2004) Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg 240: 205–213
16. Kamath PS, Wiesner RH, Malinchoc M et al (2001) A model to predict survival in patients with end-stage liver disease. Hepatology 33:464–470
17. Pugh RN, Murray-Lyon IM, Dawson JL et al (1973) Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 60:646–649
18. Benzoni E, Cojutti A, Lorenzin D et al (2007) Liver resective surgery: a multivariate analysis of postoperative outcome and complication. Langenbecks Arch Surg 392:45–54
19. Yang T, Zhang J, Lu JH et al (2011) Risk factors influencing postoperative outcomes of major hepatic resection of hepatocellular carcinoma for patients with underlying liver diseases. World J Surg 35:2073–2082. doi:10.1007/s00268-011-1161-0
20. Okamura Y, Takeda S, Fujii T et al (2011) Prognostic significance of postoperative complications after hepatectomy for hepatocellular carcinoma. J Surg Oncol 104:814–821
21. Young AL, Adair R, Prasad KR et al (2012) Hepatocellular carcinoma within a noncirrhotic, nonfibrotic, seronegative liver: surgical approaches and outcomes. J Am Coll Surg 214:174–183
22. Belli G, Fantini C, D'Agostino A et al (2004) Laparoscopic liver resections for hepatocellular carcinoma (HCC) in cirrhotic patients. HPB (Oxford) 6:236–246
23. Schneider PD (2004) Preoperative assessment of liver function. Surg Clin North Am 84:355–373
24. Hasegawa K, Kokudo N (2009) Surgical treatment of hepatocellular carcinoma. Surg Today 39:833–843
25. Manizate F, Hiotis SP, Labow D et al (2010) Liver functional reserve estimation: state of the art and relevance to local treatments. Oncology 78(Suppl 1):131–134
26. Mizuguchi T, Kawamoto M, Meguro M et al (2012) Serum antithrombin III level is well correlated with multiple indicators for assessment of liver function and diagnostic accuracy for predicting postoperative liver failure in hepatocellular carcinoma patients. Hepatogastroenterology 59:551–557
27. Goodman DS (1980) Plasma retinol-binding protein. Ann NY Acad Sci 348:378–390
28. Truant S, Boleslawski E, Duhamel A et al (2012) Tumor size of hepatocellular carcinoma in noncirrhotic liver: a controversial predictive factor for outcome after resection. Eur J Surg Oncol 38:1189–1196
29. Cucchetti A, Ercolani G, Vivarelli M et al (2006) Impact of model for end-stage liver disease (MELD) score on prognosis after hepatectomy for hepatocellular carcinoma on cirrhosis. Liver Transpl 12:966–971

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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

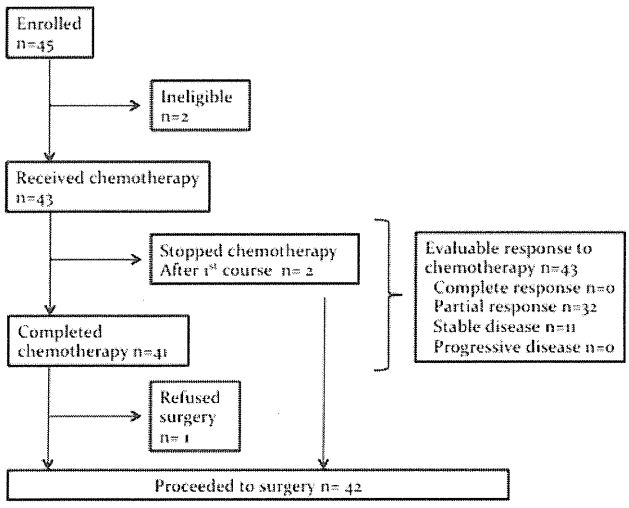

Fig. 1 Trial profile

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

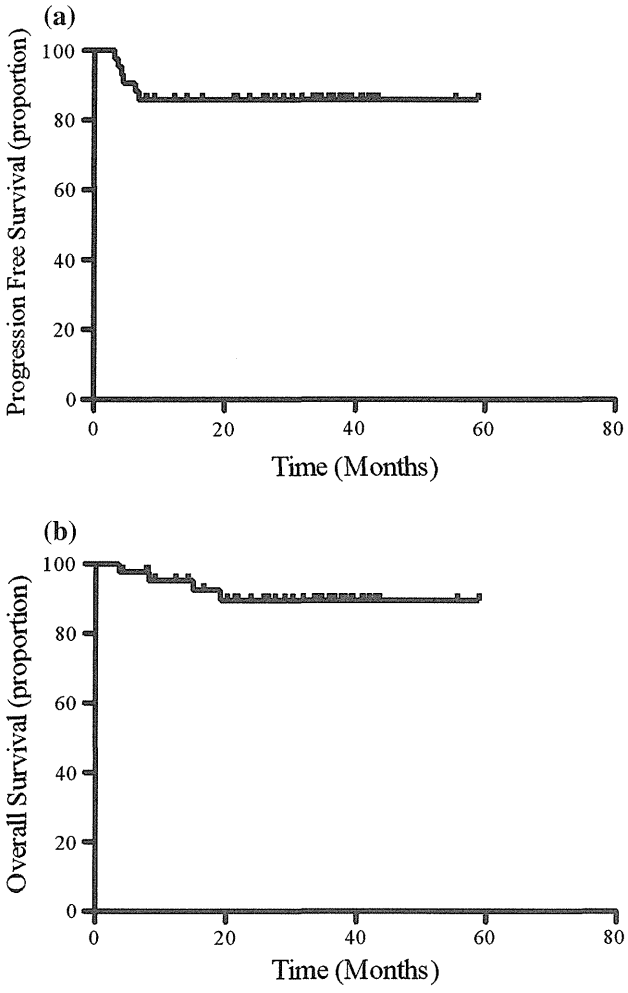

Fig. 2 Kaplan–Meier curves for **a** progression-free survival and **b** overall survival

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.

References

1. Jemal A, Center MM, DeSantis C et al (2010) Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 19:1893–1907
2. Shi Y, Zhou Y (2010) The role of surgery in the treatment of gastric cancer. J Surg Oncol 101:687–692
3. Songun I, Putter H, Kranenbarg EM et al (2010) Surgical treatment of gastric cancer: 15-year follow-up results of the randomised nationwide Dutch D1D2 trial. Lancet Oncol 11:439–449
4. Sakuramoto S, Sasako M, Yamaguchi T et al (2007) Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. N Engl J Med 357:1810–1820
5. Cunningham D, Allum WH, Stenning SP et al (2006) Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med 355:11–20
6. Ychou M, Boige V, Pignon JP et al (2011) Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase III trial. J Clin Oncol 29:1715–1721
7. Ajani JA (2005) Evolving chemotherapy for advanced gastric cancer. Oncologist 10:49–58
8. Van Cutsem E, Moiseyenko VM, Tjulandin S et al (2006) Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 study group. J Clin Oncol 24:4991–4997
9. Ajani JA, Rodriguez W, Bodoky G et al (2010) Multicenter phase III comparison of cisplatin/S-1 with cisplatin/infusional fluorouracil in advanced gastric or gastroesophageal adenocarcinoma study: the FLAGS trial. J Clin Oncol 28:1547–1553
10. Koizumi W, Narahara H, Hara T et al (2008) S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. Lancet Oncol 9:215–221
11. Takayama T, Sato Y, Sagawa T et al (2007) Phase I study of S-1, docetaxel and cisplatin combination chemotherapy in patients with unresectable metastatic gastric cancer. Br J Cancer 97:851–856
12. Sato Y, Takayama T, Sagawa T et al (2010) Phase II study of S-1, docetaxel and cisplatin combination chemotherapy in patients with unresectable metastatic gastric cancer. Cancer Chemother Pharmacol 66:721–728
13. Koizumi W, Nakayama N, Tanabe S et al (2012) A multicenter phase II study of combined chemotherapy with docetaxel, cisplatin, and S-1 in patients with unresectable or recurrent gastric cancer (KDOG 0601). Cancer Chemother Pharmacol 69(2):407–413
14. Japanese Gastric Cancer Association (1998) Japanese classification of gastric carcinoma—2nd english edition. Gastric Cancer 1:10–24
15. Japanese Gastric Cancer Association (2001) Japanese classification of gastric carcinoma—2nd english edition—response assessment of chemotherapy and radiotherapy for gastric carcinoma: clinical criteria. Gastric Cancer 4:1–8
16. Japanese Gastric Cancer Association (2011) Japanese classification of gastric carcinoma: 3rd english edition. Gastric Cancer 14:101–112
17. Kwon HC, Roh MS, Oh SY, Kim SH, Kim MC, Kim JS, Kim HJ (2007) Prognostic value of expression of ERCC1, thymidylate synthase, and glutathione S-transferase P1 for 5-fluorouracil/oxaliplatin chemotherapy in advanced gastric cancer. Ann Oncol 18(3):504–509
18. Schuhmacher CP, Fink U, Becker K et al (2001) Neoadjuvant therapy for patients with locally advanced gastric carcinoma with etoposide, doxorubicin, and cisplatinum. Closing results after 5 years of follow-up. Cancer 91:918–927
19. Yoshikawa T, Sasako M, Yamamoto S et al (2009) Phase II study of neoadjuvant chemotherapy and extended surgery for locally advanced gastric cancer. Br J Surg 96:1015–1022
20. Kinoshita T, Sasako M, Sano T et al (2009) Phase II trial of S-1 for neoadjuvant chemotherapy against scirrhous gastric cancer (JCOG 0002). Gastric Cancer 12:37–42
21. Fareed KR, Al-Attar A, Soomro IN, Kaye PV, Patel J, Lobo DN, Parsons SL, Madhusudan S (2010) Tumour regression and ERCC1 nuclear protein expression predict clinical outcome in patients with gastro-oesophageal cancer treated with neoadjuvant chemotherapy. Br J Cancer 102(11):1600–1607
22. Matsubara J, Nishina T, Yamada Y, Moriwaki T, Shimoda T, Kajiwara T, Nakajima TE, Kato K, Hamaguchi T, Shimada Y, Okayama Y, Oka T, Shirao K (2008) Impacts of excision repair cross-complementing gene 1 (ERCC1), dihydropyrimidine dehydrogenase, and epidermal growth factor receptor on the outcomes of patients with advanced gastric cancer. Br J Cancer 98(4):832–839
23. Takimoto R, MacLachlan TK, Dicker DT, Niitsu Y, Mori T, el-Deiry WS (2002) BRCA1 transcriptionally regulates damaged DNA binding protein (DDB2) in the DNA repair response following UV-irradiation. Cancer Biol Ther 1(2):177–186
24. Li W, Qin J, Sun YH et al (2010) Neoadjuvant chemotherapy for advanced gastric cancer: a meta-analysis. World J Gastroenterol 16:5621–5628
25. De Vita F, Giuliani F, Galizia G et al (2007) Neo-adjuvant and adjuvant chemotherapy of gastric cancer. Ann Oncol 18(Suppl 6):120–123
26. Mezhir JJ, Tang LH, Coit DG (2010) Neoadjuvant therapy of locally advanced gastric cancer. J Surg Oncol 101:305–314
27. Saka M, Morita S, Fukagawa T et al (2011) Present and future status of gastric cancer surgery. Jpn J Clin Oncol 41:307–313
28. Kawashima Y, Sasako M, Tsuburaya A et al (2008) Phase II study of preoperative neoadjuvant chemotherapy (CX) with S-1

plus cisplatin for gastric cancer (GC) with bulky and/or para-aortic lymph node metastases: a Japan clinical oncology group study (JCOG0405). In: ASCO gastrointestinal cancers symposium. Abstract no. 118, San Francisco
29. Fujitani K, Sasako M, Iwasaki Y et al (2007) A phase II study of preoperative chemotherapy (CX) with S-1 and cisplatin followed by gastrectomy for clinically resectable type 4 and large type 3 gastric cancer. JCOG 0210 J Clin Oncol 25:18 (suppl; abstr 4609)
30. Ajani JA, Mansfield PF, Crane CH et al (2005) Paclitaxel-based chemoradiotherapy in localized gastric carcinoma: degree of pathologic response and not clinical parameters dictated patient outcome. J Clin Oncol 23:1237–1244

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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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Key Words: Colon carcinoma, invasion, *FERMT1*, DNA microarray, fermitin family.

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

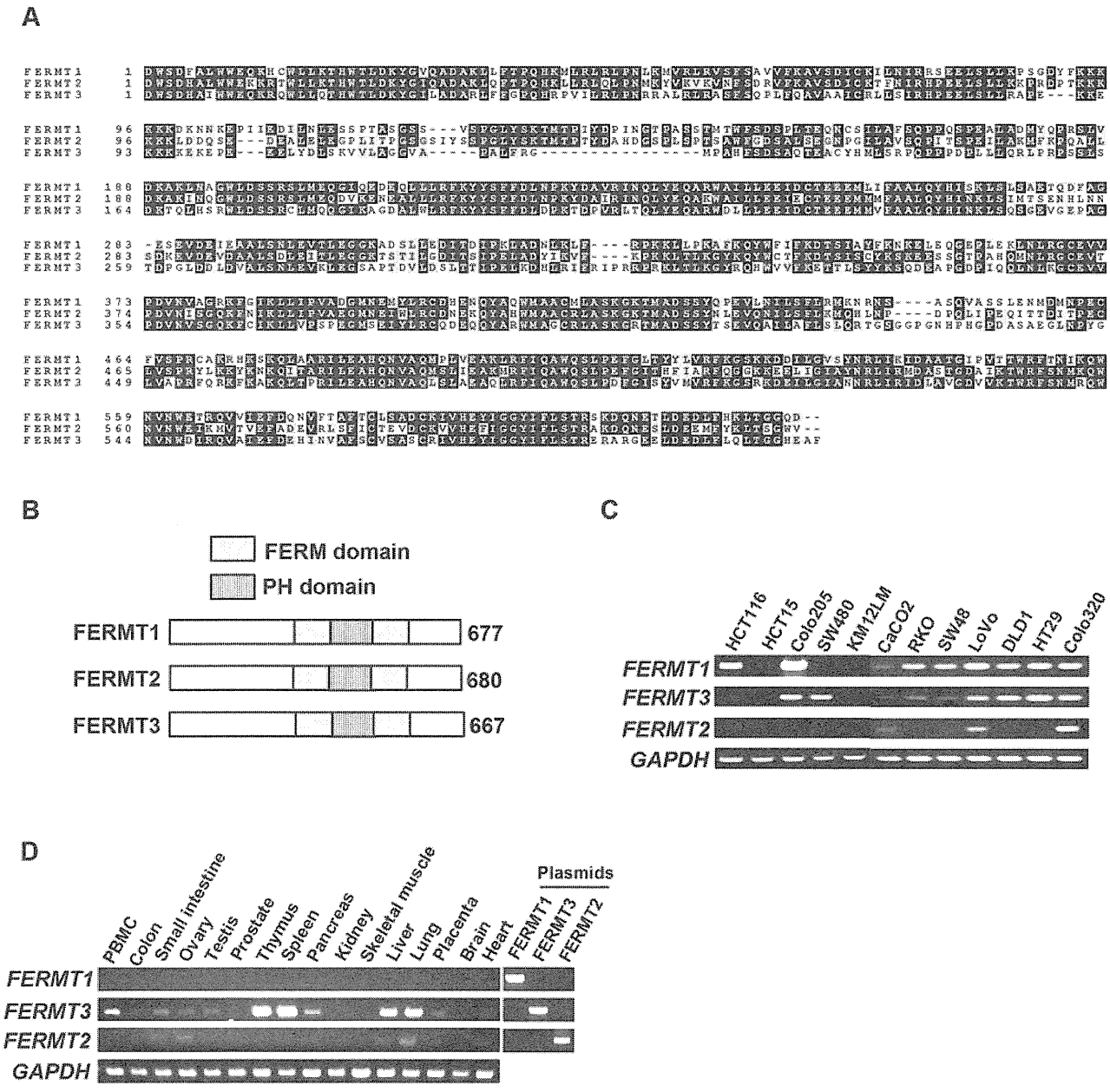

Figure 1. *Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins. A dotted box indicates the FERMT domain, cytoskeletal-associated domain, a lined box indicates the Pleckstrin homology domain (PH) domain, phosphatidylinositol lipid association domain. C: Reverse transcription-polymerase chain reaction (RT-PCR) of FERMT family in colon carcinoma cells. FERMT1, FERMT2 and FERMT3 expression in colon carcinoma cells was evaluated by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal positive control. D: RT-PCR of FERMT family genes in normal organ tissues. FERMT1, FERMT2 and FERMT3 expression in normal organ tissues was evaluated by RT-PCR. FERMT1, FERMT2 and FERMT3 plasmids were used as positive controls. GAPDH was used as an internal positive control.*

lipids association domain, respectively (Figure 1B). Since *FERMT1*, *FERMT2* and *FERMT3* show high homology with each other, we evaluated the expressions of these genes in colon carcinoma cells and also in normal organ tissues by RT-PCR. *FERMT1* was expressed in 9 (75%) out of 12 colon carcinoma line cells, and *FERMT3* was expressed in 9 (75%) out of 12 colon carcinoma line cells and *FERMT2* was expressed in 3 (25%) out of 12 colon carcinoma line cells

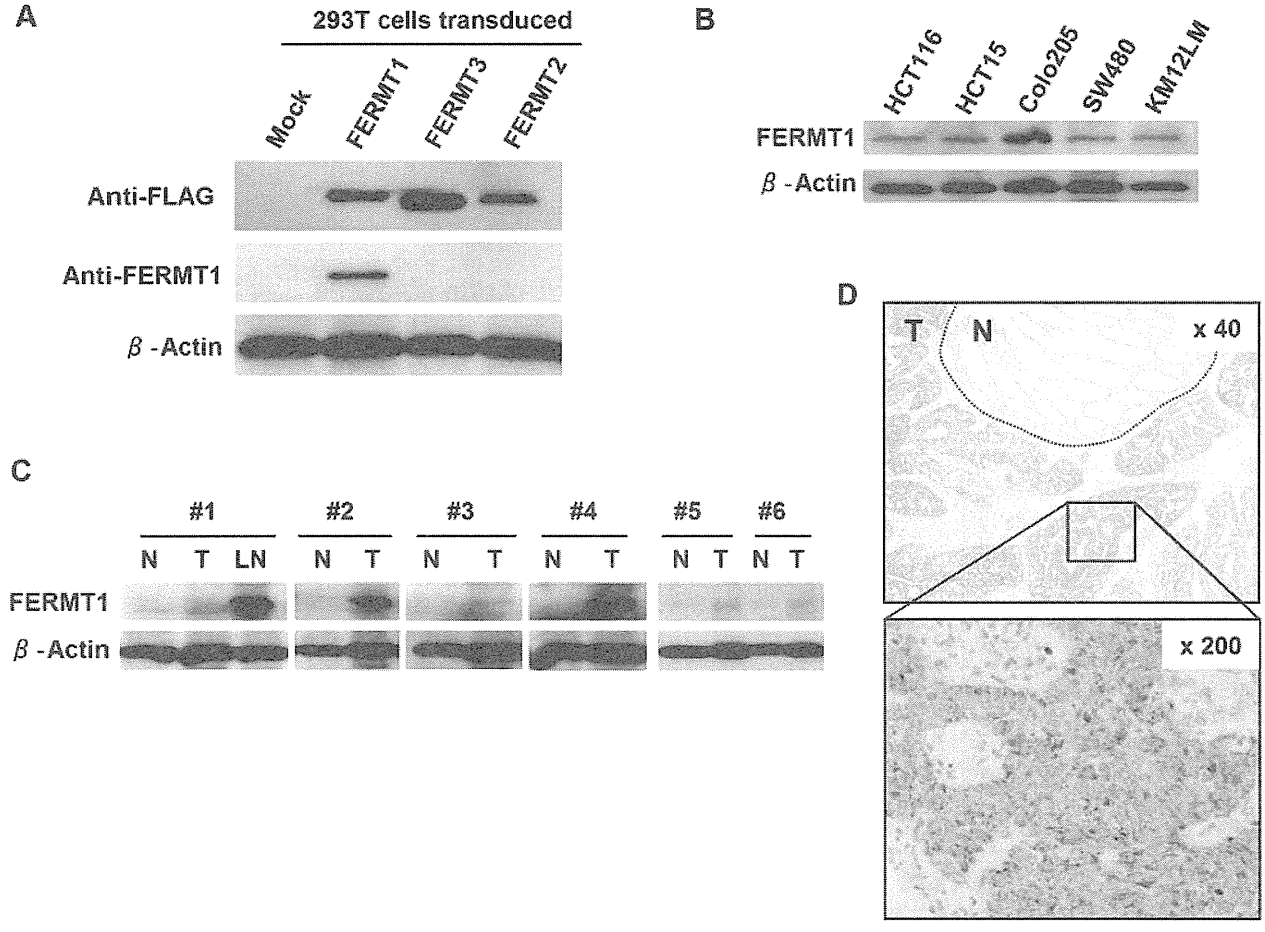

Figure 2. *Fermitin family member 1 (FERMT1) protein expression in colonic carcinomas. A: Western blotting using monoclonal antibody (mAb) against FERMT1. 293T cells were transfected with FERMT1, FERMT2 and FERMT3 plasmids. Western blotting using anti-FLAG mAb and anti-FERMT1 mAb was performed. Anti-FLAG mAb was used as a positive control. β-Actin was used as an internal positive control. B: Western blotting of colonic carcinoma cells. Western blotting using anti-FERMT1 mAb was performed. β-Actin was used as an internal positive control. C: Western blot of colon carcinoma tissues. Protein expression of FERMT1 in primary human colonic carcinoma cases (#1-#6) was evaluated by western blotting using an anti-FERMT1 mAb. T, Tumoral part of colonic carcinoma tissue; N, adjacent normal colonic mucosa tissue; LN, lymph node metastatic tissue of the corresponding case. β-Actin was used as an internal positive control. D: Immunohistochemical staining of FERMT1. Representative images of immunohistochemical staining of colonic carcinoma tissues using anti-FERMT1 mAb are shown. Brown indicates positive staining. Dotted line indicates normal colonic mucosa cells. N, Normal colon mucosa tissue; T, colonic carcinoma tissue.*

(Figure 1C). *FERMT1* was not expressed in normal organ tissues, whereas *FERMT3* and *FERMT2* were expressed ubiquitously in normal organ tissues. Only *FERMT1* exhibits colon carcinoma cell-specific expression. We therefore focused on *FERMT1* for further analysis.

Protein expression of FERMT1 in colon carcinoma cells and tissues. To address FERMT1 protein expression, we established a novel anti-FERMT1 mAb. Since FERMT1, FERMT2 and FERMT3 have similar protein structures, we evaluated the specificity of the mAb to FERMT1. FERMT1 mAb showed reactivity for 293T cells transfected with a FERMT1 expression vector, whereas it did not react to 293T cells transfected with a FERMT2 or FERMT3 vector, as shown in western blot analysis (Figure 2A), indicating that the mAb against FERMT1 mAb is specific for FERMT1. Western blot analysis revealed positive FERMT1 protein expression in all five colon carcinoma lines tested (Figure 2B).

Further evaluation of FERMT1 protein expression in primary colon carcinoma tissues was performed. Six colon carcinoma primary tumor tissues exhibited higher levels of FERMT1 protein expression than those in adjacent normal colonic mucosa tissues (Figure 2C). Of note, stronger FERMT1 protein expression was detected in tissue from lymph node metastasis of case #1 than in primary colonic tumor tissue and normal colonic mucosa of the same case.

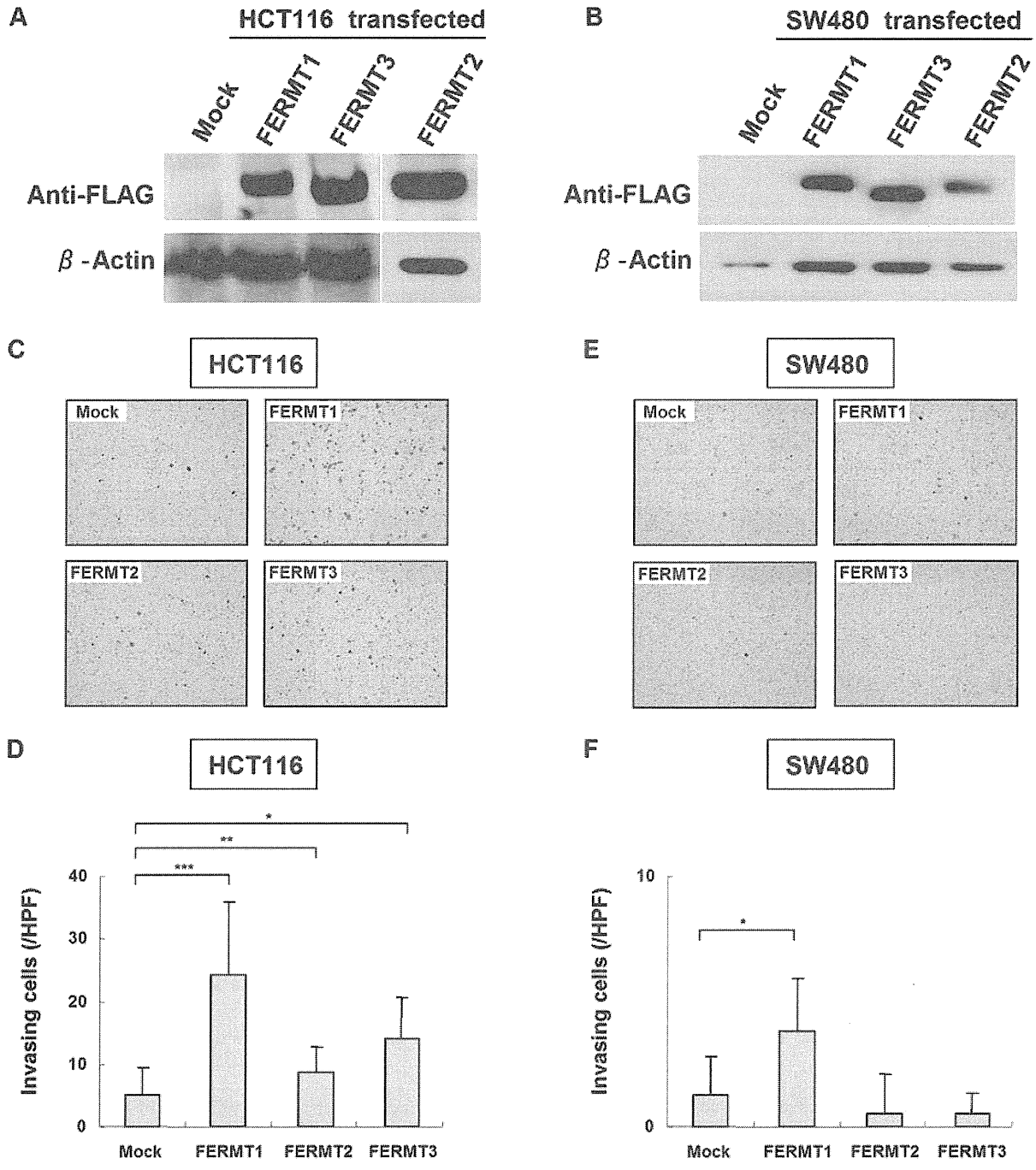

Figure 3. *Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control. B: Western blotting using a monoclonal antibody (mAb) to FLAG-tag. SW480 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using a mAb to FLAG-tag. β-Actin was used as an internal positive control. C: Invasion assay of FERMT family-overexpressing HCT116 cells. Representative images of invasion assay using FERMT family cDNA-overexpressing HCT116 cells. Purple cells indicate HCT116 cells that have invaded through the Matrigel. D: Invasion assay of FERMT family-overexpressing HCT116 cells. Invading cells were counted in 10 high power fields (HPFs). Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 cells were examined for statistical significance using the Student's t-test. *p=0.03, **p=0.001, ***p<0.0001. E: Invasion assay of FERMT family-overexpressing SW480 cells. Representative images of invasion assay using FERMT family cDNA-overexpressing SW480 cells. Purple cells indicate SW480 cells that have invaded through the Matrigel. F: Invasion assay of FERMT family-overexpressing SW480 cells. Invaded cells were counted in 10 HPF. Data represent means+SD. Differences between FERMT family-overexpressing SW480 cells and mock-transfected SW480 cells were examined for statistical significance using Student's t-test. *p=0.04.*

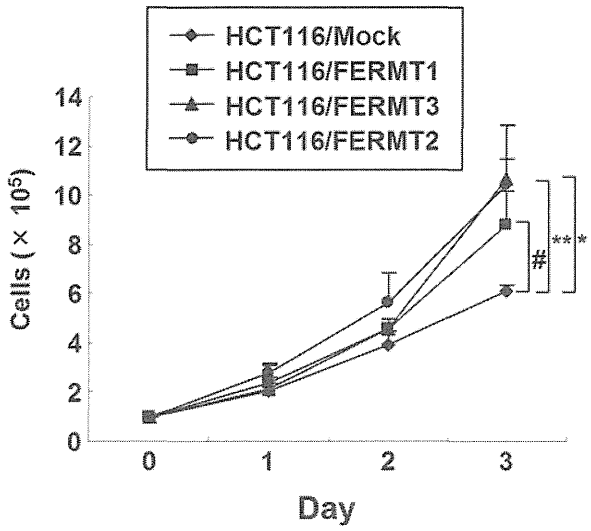

Figure 4. *Cell growth of FERMT famliy-overexpressing HCT116 cells. FERMT family cDNA-overexpressing HCT116 cells were seeded in a 6-well plate, and the cell growth rate was recorded daily. Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 cells were examined for statistical significance using Student's t-test. *p=0.015, #p=0.012, **p=0.001.*

Immunohistochemical staining of primary colonic carcinoma tissues also revealed FERMT1 protein expression in carcinoma cells but not in normal epithelial cells (Figure 2D). The positive immunohistochemical staining rate of FERMT1 protein in colon carcinoma tissues was 95% (38 out of 40 cases).

Role of FERMT1 in invasion and cell growth. Since western blot analysis revealed a high level of FERMT1 protein expression in lymph node metastasis tissue, we hypothesized that FERMT1 is related to the invasion of colonic carcinoma cells. In order to analyze the functions of *FERMT* genes, we established *FERMT1*-, *FERMT2*- and *FERMT3*-overexpressing HCT116 cells and SW480 cells. Protein expression of FERMT1, FERMT2 and FERMT3 was confirmed by western blot analysis, using an anti-FLAG antibody (Figure 3A and 3B). Invasion assays using Matrigel were performed, and FERMT1-overexpressing HCT116 cells exhibited greater invasive ability than mock vector-transformed HCT116 cells ($p<0.001$) (Figure 3C and 3D). *FERMT1*-overexpressing SW480 cells also exhibited greater invasive ability than did mock-transfected SW480 cells (Figure 3E and 3F). *FERMT2* and *FERMT3* had the ability to enhance the invasion of HCT116 cells, whereas they had no effect on SW480 cells. Cell growth ability was evaluated by a cell growth assay. *FERMT1*-, *FERMT2*- and *FERMT3*-overexpressing HCT116 cells showed greater growth *in vitro* than non-transfected cells, indicating that *FERMT1*, *FERMT2* and *FERMT3* have roles in cell growth (Figure 4).

Discussion

During cancer progression, cells gain multiple abilities allowing them to become malignant cells. Malignant diseases are defined by invasion into adjacent organs and distant metastasis, and invasion is thus a prominent ability of malignant cells. In this study, we identified *FERMT1* as a colon carcinoma-related gene by screening of a gene database. *FERMT1* was reported to be overexpressed in lung carcinoma cells and colonic carcinoma cells (4). However, the molecular functions of *FERMT1* in colonic carcinoma cells have not been elucidated. In another study, *FERMT1* was shown to be overexpressed in lung metastasis of breast carcinoma (9). The same research group reported that *FERMT1* has a role in epithelial mesenchymal transition through activation of transforming growth factor-β (TGFβ) signaling (6). However, the molecular functions of *FERMT1* have remained elusive, and we therefore analyzed *FERMT1* function in colon carcinoma cells.

FERMT1 has 80% homology with *FERMT2* and 72% homology with *FERMT3*. The three molecules have similar domain structures (Figure 1B), suggesting similar molecular functions. However, the expression profiles of *FERMT1*, *FERMT2* and *FERMT3* in normal organ tissues exhibited significant differences, and only *FERMT1* showed carcinoma cell-specific expression. In this study, we did not address the expression of *FERMT1* in skin tissue; however, previous studies showed that *FERMT1* is expressed in skin keratinocytes and that gene mutation in *FERMT1* is related to Kindler syndrome (10-12). *FERMT2* was shown to have invasion ability in MCF7 breast carcinoma cells (5). *FERMT3* was reported to be expressed in leukocytes and to have a role in the activation of integrin signals (13, 14); however, there has been no report describing the relationship between *FERMT3* and invasion. In our study, *FERMT1*, *FERMT2* and *FRMT3* were all shown to have roles in invasion, indicating that they may have similar functions. *FERMT1* and *FERMT2* have been reported to share some molecular functions in skin keratinocytes (15, 16). These observations indicate that *FERMT1*, *FERMT2* and *FERMT3* may have similar molecular functions and that the difference in expression defines the role of each molecule. Of note, *FERMT1* is ectopically and specifically overexpressed in carcinoma cells and *FERMT1* is thus the most suitable target for future cancer therapy.

In summary, to our knowledge this is the first report on *FERMT1* functions in colon carcinoma cells. While *FERMT1*, *FERMT2* and *FERMT3* are expressed in colon carcinoma cells, only *FERMT1* exhibites cancer cell-specific expression. FERMT1 also has a role in invasion and growth of colonic carcinoma cells. The results indicate that *FERMT1* is a possible target for cancer therapy.