the absence of control of liver metastasis. We have performed hepatectomy without LN dissection for ICC limited to within the peripheral region of the liver, while hepatectomy with extrahepatic bile duct resection and LN dissection both in the hepatoduodenal ligament and around the head of the pancreas were performed for any type of ICC extended to the hepatic hilum. In the present study, we evaluated the surgical outcome in ICC patients following the aforementioned therapeutic strategy, and analyzed prognostic factors influencing patient survival with respect to the manner of recurrence.

Patients and methods

Between October 1989 and January 2005, 41 patients underwent surgical resection of ICC at the Department of Surgery, Shinshu University Hospital, with no macroscopic evidence of residual cancer. The patient population comprised 24 men and 17 women with a median age of 67 years (range, 52–81 years). There were no surgery- or hospital-related deaths.

Surgical procedures included trisegmentectomy in 5 patients, extended hemihepatectomy in 22 patients, hemihepatectomy in 7 patients, and minor hepatic resection (less than one segment) in 7 patients. Among these 41 patients, concomitant portal vein resection and reconstruction were performed in 4 patients, and right hepatic artery resection/reconstruction in 1 patient. Although it is difficult to evaluate definitely the presence or absence of lymph node metastasis without LN dissection, patients whose preoperative imaging studies failed to detect lymph node metastasis were categorized as having an absence of lymph node metastasis and no indication for LN dissection. Extrahepatic bile duct resection with LN dissection, both in the hepatoduodenal ligament and around the head of the pancreas, was performed when the tumor extended into the hepatic hilum or the hepatoduodenal ligament. Vascular resection and reconstruction were also performed when needed for curative resection.

The median follow-up time was 19 months (range, 2–180 months). Initially, no patients underwent adjuvant chemotherapy. After discharge, all patients were followed up at our outpatient clinic on a monthly or bimonthly basis. When recurrence was detected by various imaging modalities, re-laparotomy was chosen for patients eligible for a second hepatectomy, and chemotherapy was chosen for patients ineligible for surgery. Up through the end of January 2005, three patients underwent a second hepatectomy for solitary tumor recurrence in the liver.

The pathological features of ICC at initial hepatectomy were evaluated according to the classification guidelines proposed by the Liver Cancer Study Group in Japan.[10] On the basis of the gross appearance of the cut surface, the ICC was categorized as either the mass-forming type, the periductal-infiltrating type, the intraductal growth type, or other. When more than one type was found, all types involved were recorded in the order of the degree of involvement. Resected specimens were also examined for several pathological parameters. The hilar type was defined as a tumor invading the portal vein, hepatic artery, or bile duct in the hepatic hilum. The peripheral type was defined as a tumor without invasion of the hepatic hilum. The hilar bile duct was defined as the portion of the bile duct comprising the right hepatic duct, left hepatic duct, and hepatic confluence. Pure periductal-infiltrating-type ICCs extending to the hepatic hilum were excluded from this study because they were indistinguishable from hilar bile duct carcinoma. As prognostic factors, patient age and sex, preoperative serum carcinoembryonic antigen (CEA) and carbohydrate antigenic determinant 19-9 (CA 19-9) levels, preoperative jaundice, tumor location in the liver, gross tumor appearance, tumor diameter, histological differentiation, vascular invasion, intrahepatic metastasis, LN metastasis at initial hepatectomy, and residual cancer at the transected margin were examined. The cutoff values at our institute for CEA and CA19-9 are 2.5 ng/ml and 37 U/ml, respectively. The CA 19-9 level is often elevated in the patients with obstructive jaundice. All patients with preoperative jaundice underwent biliary drainage, and the bilirubin level decreased to less than 2 mg/dl before surgery. We measured CA 19-9 levels in all patients when serum total bilirubin levels decreased to less than 2 mg/dl. Portal vein involvement was defined as microscopy-confirmed tumor thrombus or vascular wall invasion in any portion containing branches of the portal vein.

The Mann-Whitney U test was performed for all two-group comparisons. The χ-squared method with Yates' correction or Fisher's exact test was used for qualitative variables. The survival curves were calculated by the Kaplan-Meier method, and statistical significance was tested by the log-rank test. Variables to be entered into the multiple logistic regression analysis were chosen on the basis of the results of univariate analysis. The model selected for the multiple regression analysis was created by the backward elimination method, and the goodness of fit of the adopted model was assessed by Akaike's information criterion (AIC)[20] and the G value (difference of deviance). All analyses were performed with the Statview 5.0 statistical software package (Abacus Concepts, Berkeley, CA, USA) and the R software package (R Development Core Team, 2006; R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0; http://www.r-project.org). Differences with $P<0.05$ were considered to be statistically significant.

Results

Patient clinicopathological features are summarized in Table 1. The cumulative 1-, 3-, and 5-year survival rates after initial hepatectomy were 79.1%, 36.3%, and 28.7%, respectively. Univariate analysis showed that significant risk factors for poorer survival included preoperative jaundice ($P = 0.0115$), preoperative serum CA19-9 levels ≥37 U/ml ($P = 0.0089$), tumor diameter ≥4.5 cm (median value) ($P = 0.017$), ICC extended to the hepatic hilum ($P = 0.0065$), mass-forming with periductal-infiltrating-type ICC ($P = 0.003$), poorly differentiated adenocarcinoma, LN metastasis at initial hepatectomy ($P < 0.0001$), and a positive surgical margin ($P = 0.023$). In particular, in patients with a mass-forming type ICC <4.5 cm in diameter located in the peripheral region of the liver, the 5-year survival rate was 58.3% without LN dissection. Neither age, sex, preoperative serum CEA level, portal vein involvement, nor intrahepatic metastasis significantly correlated with patient survival after initial hepatectomy (Table 1).

Among 26 patients who underwent LN dissection at initial hepatectomy, 16 were positive for LN metastasis (61.5%). Recurrence in patients who were LN-positive

Table 1. Univariate analysis of predictive factors

Factors	Survival rate (%)		*P* value
	3-year	5-year	
Age			0.1089
<67 ($n = 18$)	51.0	43.7	
≥67 ($n = 23$)	30.7	13.3	
Sex			0.2018
Male ($n = 24$)	34.2	19.5	
Female ($n = 17$)	47.1	39.3	
CA19-9			0.0089
<37.0 U/ml ($n = 13$)	70.1	70.1	
≥37.0 U/ml ($n = 28$)	23.5	15.7	
CEA			0.6978
<2.5 ng/ml ($n = 34$)	49.5	29.1	
≥2.5 ng/ml ($n = 63$)	28.2	28.2	
Preoperative jaundice			0.0115
Absent ($n = 32$)	50.6	36.3	
Present ($n = 9$)	18.4	12.3	
Tumor location			0.0065
Hilar ($n = 24$)	18.4	12.3	
Peripheral ($n = 17$)	68.2	51.1	
Tumor diameter			0.017
<45 mm ($n = 18$)	58.9	47.1	
≥45 mm ($n = 23$)	26.5	15.9	
Tumor type			0.003
Mass ($n = 25$)	58.9	44.2	
Mass with infiltrating ($n = 16$)	13.3	6.7	
Portal vein involvment			0.0785
Absent ($n = 10$)	77.8	46.7	
Present ($n = 31$)	28.3	24.3	
Intrahepatic metastasis			0.596
Absent ($n = 33$)	40.2	28.7	
Present ($n = 8$)	37.5	25.0	
Histological differentiation			0.393
Squamous cell[a]			
Well differentiated	60.0	40.0	0.002 vs poor
Moderately differentiated	38.9	29.2	0.019 vs poor
Poorly differentiated	0	0	
Lymph node metastasis			<0.0001
Absent ($n = 25$)	68.2	48.9	
Present ($n = 16$)	0	0	
Surgical margin			0.023
Not exposed ($n = 31$)	50	35.9	
Exposed ($n = 10$)	0	0	

CEA, carcinoembryonic antien; CA19-9, carbohydrate antigenic determinant 19-9
[a] Patient has survived 26 months since surgery (at time of writing)

Table 2. Univariate analysis of predictive factors between patients with and without lymph node metastasis at initial hepatectomy

Factors	Lymph node positive	Lymph node negative	*P* value
Age			0.5396
<67	6	12	
≥67	10	13	
Sex			0.3444
Male	11	13	
Female	5	12	
CA19-9			0.0447
<37.0 U/ml	2	11	
≥37.0 U/ml	14	14	
CEA			0.1197
<2.5 ng/ml	6	16	
≥2.5 ng/ml	10	9	
Preoperative jaundice			0.1185
Absent	10	12	
Present	6	3	
Tumor location			0.0035
Hilar	14	10	
Peripheral	2	5	
Tumor diameter			0.0121
<45 mm	3	15	
≥45 mm	13	10	
Tumor type			0.3304
Mass	8	11	
Mass with infiltrating	8	8	
Portal vein involvement			0.0592
Absent	1	9	
Present	15	16	
Intrahepatic metastasis			0.2252
Absent	11	22	
Present	5	3	
Histological differentiation			0.135
Squamous cell	0	1	
Well differentiated	4	7	
Moderately differentiated	9	13	
Poorly differentiated	3	4	

at initial hepatectomy was as follows: intrahepatic recurrence in 11 patients (68.9%), peritoneal dissemination in 4 patients (25%), and bone metastasis in 1 patient (6.3%). None of the patients who underwent LN dissection at initial hepatectomy experienced LN recurrence. Patients with LN metastasis at initial hepatectomy had a significantly higher incidence of high serum CA19-9 levels (≥37 U/ml; $P = 0.0447$), ICC extended to the hepatic hilum ($P = 0.0035$), and large tumors (≥4.5 cm in diameter; $P = 0.0121$) compared with those who did not have LN metastasis (Table 2).

As of the end of January 2005, 27 patients had experienced cancer recurrence: 20 patients (74.1%) had intrahepatic recurrence, 6 (22.2%) had peritoneal dissemination, 3 (11.1%) had bone metastasis, 3 (11.1%) had LN metastasis, 2 (7.4%) had skin metastasis, and 1 (3.7%) had lung metastasis. Serosal invasion by the primary tumor was observed in three of six patients with peritoneal dissemination. All LN recurrent patients had mass-forming with periductal-infiltrating-type ICC located in the peripheral liver, and none of them had undergone LN dissection at initial hepatectomy. Patients with intrahepatic recurrence had a significantly high incidence of high serum CA19-9 levels (≥37 U/ml; $P = 0.0006$), ICC extending to the hepatic hilum ($P = 0.0349$), large tumors (≥4.5 cm; $P = 0.0351$), portal vein involvement ($P = 0.0423$), LN metastasis at initial hepatectomy ($P = 0.009$), or preoperative jaundice ($P = 0.0262$) compared with disease-free patients. In particular, all 7 patients with preoperative jaundice had intrahepatic recurrence, while 13 of the remaining 27 patients (48.1%) had intrahepatic recurrence (Table 3). The univariate logistic regression analysis showed CA19-9 levels (≥37 U/ml; $P = 0.0011$), ICC extending to the hepatic hilum ($P = 0.0215$), large tumors (≥4.5 cm; $P = 0.0265$), portal vein involvement ($P = 0.038$), and LN metastasis at initial hepatectomy ($P = 0.0145$) significantly influenced intrahepatic recurrence (Table

Table 3. Univariate analysis of predictive factors between patients with intrahepatic recurrence and these without recurrence

Factors	Intrahepatic recurrence	No recurrence	*P* value
Age			0.4876
<67	7	7	
≥67	13	7	
Sex			0.7282
Male	8	7	
Female	12	7	
CA19-9		0.0006	
<37.0 U/ml	2	10	
≥37.0 U/ml	18	4	
CEA			0.7282
<2.5 ng/ml	11	9	
≥2.5 ng/ml	9	5	
Preoperative jaundice			0.0262
Absent	13	14	
Present	7	0	
Tumor location			0.0349
Hilar	14	4	
Peripheral	6	10	
Tumor diameter			0.0351
<45 mm	5	9	
≥45 mm	15	5	
Tumor type			0.0672
Mass	10	11	
Mass with infiltrating	10	2	
Portal vein involvement			
Absent	2	6	0.0423
Present	18	8	
Intrahepatic metastasis			>0.999
Absent	16	12	
Present	4	2	
Histological differentiation			0.393
Squamous cell	0	1	
Well differentiated	6	5	
Moderately differentiated	9	8	
Poorly differentiated	5	0	
Lymph node metastasis			0.009
Absent	9	13	
Present	11	1	
Surgical margin			0.4221
Not exposed	14	12	
Exposed	6	2	

Table 4. Significant variables by univariate logistic regression analysis

Factors	Odds	*P* value	AIC
Ca19-9 ≥ 37.0 U/ml	22.50	0.0011	35.676
Tumor location: hilar	2.995	0.0251	44.234
Tumor diameter ≥45 mm	5.400	0.0265	44.743
Portal vein involvment: positive	6.750	0.038	45.049
Lymph node metastasis: present	15.889	0.0145	40.651

4). The odds ratio of CA19-9 increased significantly from 22.5 to 30, when preoperative jaundice was added to CA19-9 in the multiple logistic regression analysis. On the other hand, the odds ratio of the other factors decreased significantly, or did not change, when preoperative jaundice was added to each in the multiple logistic regression analysis. This indicated that preoperative jaundice was a confounding factor of CA19-9. Therefore, the five independent factors were assessed by the multiple regression analysis. The multiple logistic regression analysis by the backward elimination method showed that no combination of these five factors was statistically significant. However, the combination of CA19-9 and portal vein involvement showed that the *P* values of CA19-9 and portal vein involvement were 0.00176 and 0.0512, indicating that portal vein involvement had some influence on intrahepatic recurrence. The AIC and G value were calculated to obtain the

optimum (i.e., simplest effective) model. When the models CA19-9 + jaundice and CA19-9 + portal vein involvement were compared, the AIC of the former was lower. Even when portal vein involvement was entered into the model CA19-9 + jaundice, the G value of this model was not significant compared with that of the model CA19-9 + jaundice. Thus, the optimum model in the present setting consisted of preoperative CA19-9 levels and preoperative jaundice (Table 5).

Discussion

Locoregional extension was usually advanced at the time of diagnosis, resulting in low resectability rates and poor prognosis.[7] The biological behavior of the tumor and its extrahepatic extensions limit the efficacy of surgical procedures, but aggressive surgical resection, when feasible, is currently the only definitive treatment for this tumor type.[21,22]

In the current study, the 5-year survival rate of patients who underwent surgery was 28.7%. Among the 31 patients with a negative surgical margin, the 5-year survival rate was 35.9%, which emphasizes the importance of obtaining a negative surgical margin. Particularly in patients with a mass-forming ICC < 4.5 cm in diameter located peripherally in the liver, the 5-year survival rate was 58.3% in the absence of LN dissection, reinforcing the importance of early detection of ICC for improving the prognosis of ICC patients, and indicating that LN dissection at initial hepatectomy may not be necessary in patients with a mass-forming-type ICC < 4.5 cm in diameter located in the peripheral liver.

LN metastasis has been reported to be a distinctive prognostic factor for ICC,[23–25] and this was supported by the results of the present study. However, it should be noted that all patients who underwent LN dissection at initial hepatectomy had no LN recurrence, even though 61.5% of patients undergoing LN dissection had positive LN metastasis at initial hepatectomy. While this finding is intriguing, it would be precipitous to interpret the present result as efficacy of regional LN dissection for preventing further spreading of LN metastasis beyond the regional LN in patients whose tumor extended into the hepatic hilum or hepatoduodenal ligament, because predominant intrahepatic recurrence of ICC might eclipse other concomitant or subsequent manners of recurrence. LN recurrence was detected in three patients, who belonged to the group of five patients with tumors categorized as mass-forming with periductal-infiltrating-type ICC located in the peripheral liver. This result indicates that LN dissection at initial hepatectomy might be necessary to improve the prognosis of patients who have mass-forming with periductal-infiltrating-type ICC located in the peripheral liver.[26,27] Computed tomography, magnetic resonance imaging, and direct cholangiography were utilized to preoperatively evaluate tumor extension and gross appearance.

The present study revealed that the most obvious recurrence pattern was intrahepatic recurrence, which could be predicted preoperatively by a combination of elevated serum CA19-9 levels and manifestation of obstructive jaundice. The relationship between intrahepatic recurrence and obstructive jaundice, which was a confounding factor, might possibly be explained as follows. Obstructive jaundice occurs only when a large mass-forming ICC is localized to the hepatic hilum or a periductal-infiltrating ICC extends to the hepatic confluence from an intrahepatic segmental duct, and such an advanced-stage ICC could easily metastasize. Moreover, cholestasis itself, particularly of bile acid, has been reported to directly influence cholangiocarcinoma growth through cyclooxygenase-2.[28–33] Together, advanced-stage ICC and direct influence of cholestasis might additively or synergistically accelerate intrahepatic recurrence. Additionally, the results of this study indicate that patients who have both elevated serum CA19-9 and jaundice preoperatively might be eligible for adjuvant chemotherapy, including that administered via transcatheter arterial infusion to prevent intrahepatic recurrence after curative hepatectomy, on the condition that such chemotherapy is beneficial. However, the validity of adjuvant chemotherapy has thus far been controversial. Some studies have reported promising

Table 5. AIC and G value by logistic regression analysis

Factors	AIC	Deviance	G value
CA19-9	35.676	31.676	0
CA19-9 + portal vein involvement	33.383	27.383	–4.293*
CA19-9 + jaundice	30.697	24.947	–6.729**
CA19-9 + jaundice + portal vein involvement	30.327	22.327	–2.620***

AIC, Akaike's information criterion
* Difference of deviance between CA19-9 and CA19-9 + portal vein involvement, $P < 0.05$
** Difference of deviance between CA19-9 and CA19-9 + jaundice, $P < 0.01$
*** Difference of deviance between CA19-9 + jaundice and CA19-9 + jaundice + portal vein involvement, $P > 0.1$

efficacy with respect to the response rate of gemcitabine in ICC, while others have reported a lack of change in the survival rate following surgery, even when adjuvant chemotherapy and irradiation were administered.[34-36] Evaluation of adjuvant chemotherapy based on a prospective and randomized data analysis on a multi-institutional level is mandatory to establish the best therapeutic strategy for ICC.

References

1. Yamamoto J, Kosuge T, Takayama T, Shimada K, Makuuchi M, Yoshida J, et al. Surgical treatment of intrahepatic cholangiocarcinoma: four patients surviving more than five years. Surgery 1992;111:617–22.
2. Yamanaka N, Okamoto E, Ando T, Oriyama T, Fujimoto J, Furukawa K, et al. Clinicopathologic spectrum of resected extraductal mass-forming intrahepatic cholangiocarcinoma. Cancer 1995;76:2449–56.
3. El Rassi ZE, Partensky C, Scoazec JY, Henry L, Lombard-Bohas C, Maddern G. Peripheral cholangiocarcinoma: presentation, diagnosis, pathology and management. Eur J Surg Oncol 1999;25:375–80.
4. Yamamoto M, Takasaki K, Yoshikawa T. Lymph node metastasis in intrahepatic cholangiocarcinoma. Jpn J Clin Oncol 1999;29:147–50.
5. Inoue K, Makuuchi M, Takayama T, Torzilli G, Yamamoto J, Shimada K, et al. Long-term survival and prognostic factors in the surgical treatment of mass-forming type cholangiocarcinoma. Surgery 2000;127:498–505.
6. Nozaki Y, Yamamoto M, Ikai I, Yamamoto Y, Ozaki N, Fujii H, et al. Reconsideration of the lymph node metastasis pattern (N factor) from intrahepatic cholangiocarcinoma using the International Union Against Cancer TNM staging system for primary liver carcinoma. Cancer 1998;83:1923–9.
7. Ahrendt SA, Cameron JL, Pitt HA. Current management of patients with perihilar cholangiocarcinoma. Adv Surg 1996;30:427–52.
8. Nakajima T, Kondo Y, Miyazaki M, Okui K. A histopathologic study of 102 cases of intrahepatic cholangiocarcinoma: histologic classification and modes of spreading. Hum Pathol 1988;19:1228–34.
9. Sasaki A, Aramaki M, Kawano K, Morii Y, Nakashima K, Yoshida T, et al. Intrahepatic peripheral cholangiocarcinoma: mode of spread and choice of surgical treatment. Br J Surg 1998;85:1206–9.
10. Liver Cancer Study Group of Japan. General rules for the clinical and pathological study of primary liver cancer. 2nd English ed. Tokyo: Kanehara; 2003. p. 16–7.
11. Miwa S, Miyagawa S, Soeda J, Kawasaki S. Matrix metalloproteinase-7 expression and biologic aggressiveness of cholangiocellular carcinoma. Cancer 2002;94:428–34.
12. Takagi S, Miyagawa S, Ichikawa E, Soeda J, Miwa S, Miyagawa Y, et al. Dendritic cells, T-cell infiltration, and Grp94 expression in cholangiocellular carcinoma. Hum Pathol 2004;35:881–6.
13. Okabayashi T, Yamamoto J, Kosuge T, Shimada K, Yamasaki S, Takayama T, et al. A new staging system for mass-forming intrahepatic cholangiocarcinoma: analysis of preoperative and postoperative variables. Cancer 2001;92:2374–83.
14. Inoue K, Makuuchi M, Takayama T, Torzilli G, Yamamoto J, Shimada K, et al. Long-term survival and prognostic factors in the surgical treatment of mass-forming type cholangiocarcinoma. Surgery 2000;127:498–505.
15. Uenishi T, Hirohashi K, Kubo S, Yamamoto T, Yamazaki O, Kinoshita H. Clinicopathological factors predicting outcome after resection of mass-forming intrahepatic cholangiocarcinoma. Br J Surg 2001;88:969–74.
16. Suzuki S, Sakaguchi T, Yokoi Y, Okamoto K, Kurachi K, Tsuchiya Y, et al. Clinicopathological prognostic factors and impact of surgical treatment of mass-forming intrahepatic cholangiocarcinoma. World J Surg 2002;26:687–93.
17. Aishima S, Asayama Y, Taguchi K, Sugimachi K, Shirabe K, Shimada M, et al. The utility of keratin 903 as a new prognostic marker in mass-forming-type intrahepatic cholangiocarcinoma. Mod Pathol 2002;15:1181–90.
18. Ohtsuka M, Ito H, Kimura F, Shimizu H, Togawa A, Yoshidome H, et al. Results of surgical treatment for intrahepatic cholangiocarcinoma and clinicopathological factors influencing survival. Br J Surg 2002;89:1525–31.
19. Shimada M, Yamashita Y, Aishima S, Shirabe K, Takenaka K, Sugimachi K. Value of lymph node dissection during resection of intrahepatic cholangiocarcinoma. Br J Surg 2001;88:1463–6.
20. Akaike H. Information theory and an extension of the maximum likelihood principle. In: Petrov B, Csaki H, editors. Second International Symposium on Information Theory. Budapest: Akademiai Kaido, 1973:267–81.
21. Roayaie S, Guarrera JV, Ye MQ, Thung SN, Emre S, Fishbein TM, et al. Aggressive surgical treatment of intrahepatic cholangiocarcinoma: predictors of outcomes. J Am Coll Surg 1998;187:365–72.
22. Lieser MJ, Barry MK, Rowland C, Ilstrup DM, Nagorney DM. Surgical management of intrahepatic cholangiocarcinoma: a 31-year experience. J Hepatobiliary Pancreat Surg 1998;5:41–7.
23. Kawarada Y, Yamagiwa K, Das BC. Analysis of the relationships between clinicopathologic factors and survival time in intrahepatic cholangiocarcinoma. Am J Surg 2002; 183:679–85.
24. Weber SM, Jarnagin WR, Klimstra D, DeMatteo RP, Fong Y, Blumgart LH. Intrahepatic cholangiocarcinoma: resectability, recurrence pattern, and outcomes. J Am Coll Surg 2001;193:384–91.
25. Nakagawa T, Kamiyama T, Kurauchi N, Matsushita M, Nakanishi K, Kamachi H, et al. Number of lymph node metastases is a significant prognostic factor in intrahepatic cholangiocarcinoma. World J Surg 2005;29:728–33.
26. Sasaki A, Kawano K, Aramaki M, Ohno T, Tahara K, Kitano S. Correlation between tumor size and mode of spread in mass-forming intrahepatic cholangiocarcinoma. Hepatogastroenterology 2004;51:224–8.
27. Morimoto Y, Tanaka Y, Ito T, Nakahara M, Nakaba H, Nishida T, et al. Long-term survival and prognostic factors in the surgical treatment for intrahepatic cholangiocarcinoma. J Hepatobiliary Pancreat Surg 2003;10:432–40.
28. Marucci L, Baroni GS, Mancini R, Benedetti A, Jezequel AM, Orlandi F. Cell proliferation following extrahepatic biliary obstruction. Evaluation by immunohistochemical methods. J Hepatol 1993;17:163–9.
29. Polimeno L, Azzarone A, Zeng QH, Panella C, Subbotin V, Carr B, et al. Cell proliferation and oncogene expression after bile duct ligation in the rat: evidence of a specific growth effect on bile duct cells. Hepatology 1995;21:1070–8.
30. Yoon JH, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. Gastroenterology 2002;122:985–93.
31. Han C, Leng J, Demetris AJ, Wu T. Cyclooxygenase-2 promotes human cholangiocarcinoma growth: evidence for cyclooxygenase-2-independent mechanism in celecoxib-mediated induction of p21waf1/cip1 and p27kip1 and cell cycle arrest. Cancer Res 2004;64:1369–76.
32. Han C, Wu T. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. J Biol Chem 2005;280:24053–63.

33. Knox JJ, Hedley D, Oza A, Feld R, Siu LL, Chen E, et al. Combining gemcitabine and capecitabine in patients with advanced biliary cancer: a phase II trial. J Clin Oncol 2005;23:2332–8.
34. Tanaka N, Yamakado K, Nakatsuka A, Fujii A, Matsumura K, Takeda K. Arterial chemoinfusion therapy through an implanted port system for patients with unresectable intrahepatic cholangiocarcinoma—initial experience. Eur J Radiol 2002;41:42–8.
35. Valverde A, Bonhomme N, Farges O, Sauvanet A, Flejou JF, Belghiti J. Resection of intrahepatic cholangiocarcinoma: a Western experience. J Hepatobiliary Pancreat Surg 1999;6:122–7.
36. Casavilla FA, Marsh JW, Iwatsuki S, Todo S, Lee RG, Madariaga JR, et al. Hepatic resection and transplantation for peripheral cholangiocarcinoma. J Am Coll Surg 1997;185:429–36.

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Efficacy of S-1 for Patients with Peritoneal Metastasis of Gastric Cancer

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Key Words
S-1 · Cancer, gastric · Metastasis, peritoneal · Thymidine phosphorylase

Abstract
Background: This study was designed to examine the efficacy and compliance of S-1 for the patients with peritoneal metastasis of gastric cancer. ***Methods:*** Sixteen consecutive patients with peritoneal metastasis of gastric cancer were treated with S-1. Their survival was compared with that of the historical control group (25 patients). Thymidylate synthase, dihydropyrimidine dehydrogenase, thymidine phosphorylase and orotate phosphoribosyl transferase mRNA expression in the tumor were evaluated. ***Results:*** The median survival time of S-1-treated patients was 550 days, which was significantly longer than that of the historical control group (215 days). We elucidated some factors to prolong the survival of the patients treated with S-1 for peritoneal metastasis: peritoneal metastasis without other distant metastases, the combination of S-1 treatment and gastrectomy, and low expression of thymidine phosphorylase mRNA in primary tumors. ***Conclusions:*** S-1 showed a surprisingly long-term survival with minimum toxicity in patients with peritoneal metastasis of gastric cancer.

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Introduction

Peritoneal metastasis is a predominant metastatic pattern in advanced gastric cancer [1, 2], and the prognosis of patients with peritoneal metastasis of gastric cancer is poor [3]. The median survival time (MST) of such patients is reported to be 3–6 months [4], and a standard regimen against peritoneal metastasis of gastric cancer has not yet been established.

S-1 was introduced into clinical practice in 1999, but there are only a few reports about the efficacy of S-1 for peritoneal metastasis of gastric cancer [4–6]. S-1, an oral antitumor agent, was designed based on the theory of the biochemical modulation of 5-fluorouracil (5-FU) [7–10]. In S-1, tegafur (FT) is combined with two classes of enzyme inhibitor, 5-chloro-2,4-dihydroxypyrimidine (CDHP) and potassium oxonate (Oxo), at molar ratios of 1:0.4:1 [11]. FT is a prodrug of 5-FU, and CDHP is a reversible competitive inhibitor of an enzyme involved in the degradation of 5-FU, and consequently increases antitumor activity. On the other hand, Oxo inhibits the phosphorylation of 5-FU and possibly decreases 5-FU-induced gastrointestinal tract (GI) toxicity, since GI toxicity is caused by the phosphorylation of 5-FU [11]. 5-FU is an analogue of uracil and is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). These metabolites disrupt DNA synthesis and the action of thy-

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Table 1. Characteristics of the patients

Factors	S-1 group	Control group	p value
Patients	16	25	
Age, years	62.3 ± 2.5	61.2 ± 2.8	NS
Range	38–77	25–79	
Sex			
Male	10	18	NS
Female	6	7	
Histology			
Intestinal type	4	5	NS
Diffuse type	12	20	
Primary tumor			
T1/T2/T3/T4	0/0/12/4	0/1/17/7	NS
Metastasis site (except peritoneal metastasis)			
Liver	4	5	
Lymph node	15	22	NS
Lung	0	1	
Surgical treatment			
Palliative gastrectomy (TG/DG)	13 (7/6)	17 (12/5)	
Bypass	0	2	NS
Probe laparotomy	1	5	
None	2	1	

TG = Total gastrectomy; DG = distal gastrectomy; NS = not significant.

midylate synthase (TS), and finally show anticancer effects [12–14]. Other determinants of 5-FU chemosensitivity include the 5-FU-catabolyzing enzyme, dihydropyrimidine dehydrogenase (DPD), and 5-FU-anabolyzing enzymes, such as orotate phosphoribosyl transferase (OPRT), uridine phosphorylase (UP), and thymidine phosphorylase (TP) [15–18]. Therefore, the expression level of these enzymes may reflect the sensitivity of S-1 for gastric cancer. It would be useful to clarify subgroups with high sensitivity to S-1, since such knowledge should improve the efficacy of treatment for peritoneal metastasis of gastric cancer.

In this paper, we show a surprising survival (MST: 550 days) in patients who had peritoneal metastasis of gastric cancer and were treated with S-1. In addition, we attempted to elucidate some prognostic factors including tumor-related enzyme expression levels, which would be advantageous in the treatment of peritoneal metastasis of gastric cancer with S-1.

Patients and Methods

Patient Criteria

Between August 2000 and May 2005, 16 consecutive patients with peritoneal metastasis of gastric cancer were treated with S-1 at Shinshu University Hospital. The eligibility criteria were as follows: (1) age ≥ 20 and ≤ 80 years, (2) histologically documented gastric cancer and histologically or cytologically proven peritoneal metastasis of gastric cancer, (3) no prior chemotherapy, (4) performance status of the World Health Organization (WHO) 0–2, (5) adequate organ function (white blood cell count between 4,000 and 12,000/mm^3, absolute neutrophil count of over 2,000/mm^3, platelet count of over 100,000/mm^3, hemoglobin over 9.5 g/dl, transaminase level within twice the upper limit, serum bilirubin level under 1.5 mg/dl, blood urea nitrogen under 25 mg/dl and serum creatinine under 1.5 mg/dl), (6) no serious complications, and (7) no known allergy to 5-FU. Written informed consent was obtained from all the patients and the study was approved by the institutional ethics committees.

Characteristics of the Patients

Table 1 shows the clinical features of the S-1-treated group and the historical control group. Thirteen of the S-1-treated patients had a pretreatment WHO performance status of 0, and the other 3 had a performance status of 1. Palliative gastrectomy for obstruction and/or bleeding (without lymph node dissection) was performed in 13 patients before S-1 treatment. The period of time between operation and starting to take the S-1 was 35 ± 7.2 days. The number of S-1 treatment cycles were 6.2 ± 1.7 courses (range 1–27 courses). After failure of S-1 treatment, second-line chemotherapy was administered to 4 patients (docetaxel and/or cisplatin).

All patients both of the S-1-treated group and the historical control group had histologically or cytologically proven peritoneal metastasis. In the S-1-treated group, other metastatic sites were the liver and lymph node, and the primary tumor had invaded the pancreas (T4) in 4 patients. In the historical control group, the other metastatic sites were the liver, lung, and lymph nodes, and the pri-

mary tumor had invaded the liver, pancreas or transverse colon (T4) in 7 patients.

No statistically significant difference was seen for any of the patient factors between the S-1-treated and historical control group.

Chemotherapy Regimens

S-1 was given orally twice daily after meals for 28 days, followed by 14 days' rest, as one course. Three doses of S-1 were established according to the body surface area (BSA) as follows: BSA <1.25 m^2: 80 mg/day; BSA >1.25 and <1.5 m^2: 100 mg/day, and BSA >1.5 m^2: 120 mg/day. The treatment was temporarily discontinued or the dose was reduced from 120 to 100 mg/day or from 100 to 80 mg/day, respectively, in patients with hematological toxicity of grade 3–4 or nonhematological toxicity of grade 2 or more. Treatment was continued until disease progress or unacceptable toxicity occurred or the patients chose to discontinue the treatment. All patients received S-1 in an outpatient setting.

Evaluation

Baseline evaluation included complete medical history, physical examination, complete blood cell count, serum chemistry, serum tumor marker, gastroscopy, computed tomography scans of the chest and abdomen, abdominal ultrasonography, and chest X-ray. Due to the difficulty in evaluating the responses to disseminated lesions, the primary endpoint was set as overall survival and MST, and the secondary endpoint was toxicity and compliance with the treatment. The clinicopathologic factors were classified according to the Japanese Classification of Gastric Cancer (Japanese Research Society for Gastric Cancer). Overall survival for all patients was defined as the time from the start of S-1 treatment until death or final follow-up time, using the Kaplan-Meier method. The survival of the S-1-treated group was compared with a historical control group, which had not been treated with S-1. The historical control group consisted of 25 consecutive patients with peritoneal metastasis of gastric cancer treated at the Shinshu University Hospital during 1995 and 2000. All patients conformed to the eligibility criteria similar to those described for S-1-treated patients in 'Patient Criteria'. Most control patients were treated with 5-FU, mitomycin and/or methotrexate (MTX).

A complete blood cell count, blood chemistry, and subjective/objective symptoms of toxicity were monitored on a 2-weekly basis during the treatment. Toxicity was assessed before each course according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) [19].

Quantification of mRNA Expression of Tumor-Related Enzymes

We quantified the mRNA expressions of the four tumor-related enzymes, TS, DPD, TP and OPRT, in 13 cases. A representative formalin-fixed, paraffin-embedded tumor sample from the primary tumor was selected after examination of the HE-stained slides. These tumor samples were obtained prior to the start of chemotherapy. Sections of 10-μm thickness were stained with nuclear fast red to enable visualization of histology for laser capture microdissection (PALM Microlaser Technologies, Munich, Germany), which was performed to ensure that only tumor cells were examined. Microdissected samples were collected into a microcentrifuge tube.

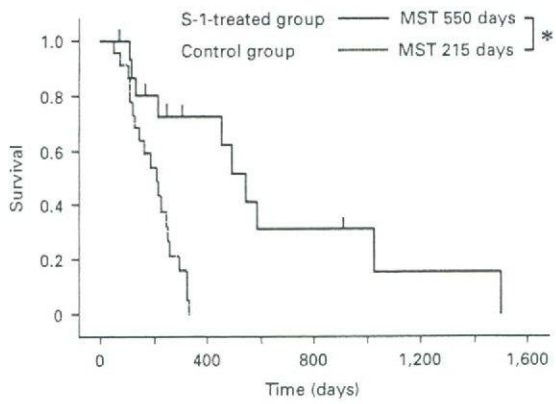

Fig. 1. Overall survival. * $p < 0.001$.

RNA extraction and cDNA synthesis were performed following the protocol of Response Genetics (Los Angeles, Calif., USA). RNA extraction was done according to a proprietary procedure (US patent No. 6,248,535). This newly developed method for the extraction of RNA from paraffin-embedded specimens now permits quantitative and accurate measurement of gene expression [20–22]. Reverse transcription was performed at 39°C for 45 min using 400 U of MMLV reverse transcriptase, 1× first strand buffer, 0.04 μg/μl random hexamers, 10 m*M* DTT, and 1 m*M* deoxynucleoside triphosphate.

Target cDNA sequences were amplified by quantitative PCR using an ABI PRISM 7900 Sequence Detection System (Taqman; Applied Biosystems, Foster City, Calif., USA) as described previously [23]. Polymerase chain reaction was performed for each gene of interest, and β-actin was used as an internal reference gene. The relative gene expression of TS, DPD, OPRT and TP was determined based on the threshold cycles of each gene in relation to the threshold cycle of the corresponding internal standard β-actin [24].

Statistics

Cumulative survival rates were calculated with the Kaplan-Meier method and compared with the log-rank test. The background of patients in the S-1-treated group and historical control group was compared with the Mann-Whitney U test or χ^2 test. A p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Survival

The overall survival curves for all patients are shown in figure 1. The MST of all patients was 550 days (18 months). One-, 2-, and 3-year survival rates were 53.8,

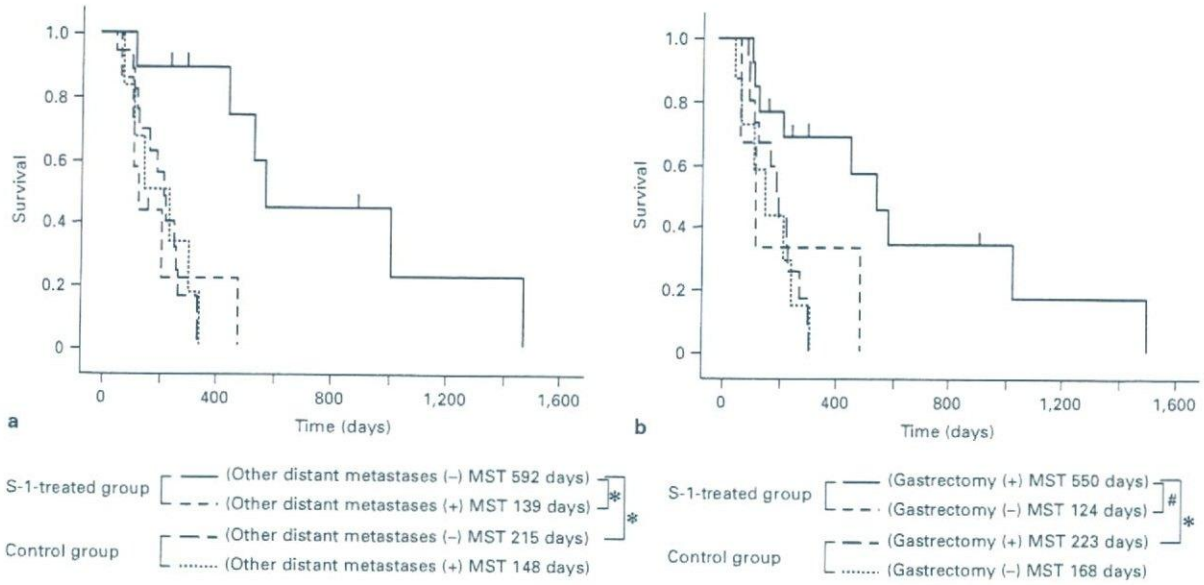

Fig. 2. a Comparison of survival between patients with peritoneal metastasis without other distant metastases and both peritoneal and other distant metastases. **b** Comparison of survival between the patients with and without gastrectomy. * $p < 0.01$; # $p < 0.05$.

23.1 and 8.1%, respectively. There were 3 patients who survived for longer than 2 years, and 1 of them survived for over 4 years. On the other hand, the MST of the historical control group was 215 days. The difference in the MST was statistically significant between the S-1-treated group and the controls ($p < 0.001$).

In addition to peritoneal metastasis, 6 patients in the S-1-treated group had other distant metastases (4 liver and 2 para-aortic lymph node metastasis, DM group). The MST of the DM group was 139 days and was significantly shorter than that of patients without other distant metastases (non-DM group; MST: 592 days, $p < 0.01$; fig. 2a). In the control group, on the other hand, there was no significant difference in the MST between the DM and non-DM group. The MST of the S-1-treated group was significantly longer than that of the control group when compared with each non-DM group.

The MST was compared between the patients who underwent a gastrectomy (OP group) and those without a gastrectomy (non-OP group). The MST was significantly higher in the OP group (550 days) than in the non-OP group (124 days) in S-1-treated patients ($p < 0.05$; fig. 2b), whereas in the control group, the MST of the OP group and non-OP-group was not statistically significant. In patients who underwent gastrectomy, the MST of the S-1-treated group was significantly longer than that of the historical control group.

Table 2. Toxicities in patients treated with S-1

	Grade				Incidence	
	1	2	3	4	total	%
Leukopenia	3	3	1		7	43.8
Anemia	3	1			4	25.0
Thrombocytopenia	1	1			2	12.5
Liver dysfunction	2	1			3	18.8
Nausea	5				5	31.3
Diarrhea	3	2			5	31.3
Skin reaction	3				3	18.8
Eye toxicity	1				1	6.3

Toxicity

Table 2 summarizes the toxicity observed during all the treatment courses. Toxicity was generally mild: grade

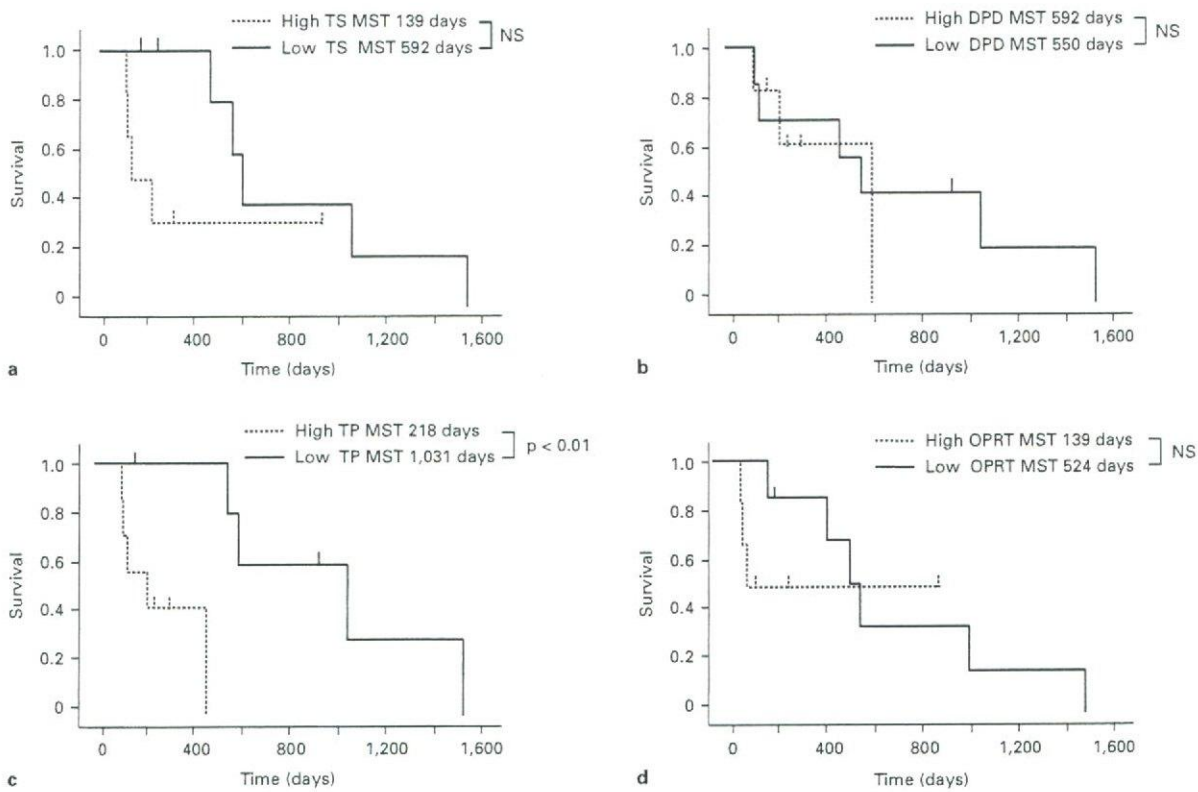

Fig. 3. Relationship between survival and mRNA expression levels of TS (**a**), DPD (**b**), TP (**c**) and OPRT (**d**). NS = Nonsignificant.

3 toxicities were observed in 1 patient and no grade 4 toxicities were observed at all. The major incidence of adverse reactions was 43.8% for leukopenia, 31.3% for nausea and diarrhea, 25.0% for anemia, 18.8% for liver dysfunction and skin reaction. There were 2 patients who died within 30 days after completion of the treatment due to tumor progression, and no treatment-related deaths occurred.

TS, DPD, TP and OPRT Expression Levels

TS, DPD, TP and OPRT mRNA expression levels were measured in 13 patients. The median TS expression, relative to the expression of the internal control housekeeping gene β-actin, was 2.5 (range 0.82–11.01). Similarly, the median DPD expression was 0.8 (range 0.53–1.87). The median TP expression was 8.0 (range 2.09–15.22), and the median OPRT expression was 1.1 (range 0.33–3.33). Overall survival was retrospectively evaluated according to the mRNA expression levels of these enzymes (TS, DPD, TP and OPRT) (fig. 3). Cutoff levels of TS, DPD, TP and OPRT were set at the median values described above. MST was significantly worse in the 6 patients with a high TP level (MST = 218 days) than in the 7 patients with a low TP level (MST = 1,031 days). No significant difference was observed in survival according to the expression of other enzymes (TS, DPD and OPRT). No significant difference was observed in the incidence of adverse events according to the enzyme mRNA expression level.

Discussion

The prognosis of patients with peritoneal metastasis of gastric cancer is poor, and a standard regimen against peritoneal metastasis of gastric cancer has not yet been

established [3, 25]. Combinations of conventional agents, such as FAMTX (fluorouracil/doxorubicin/methotrexate), MTX/5-FU (methotrexate/fluorouracil), FAM (fluorouracil/doxorubicin/mitomycin) and FP (fluorouracil/cisplatin), have been employed for treatment of such patients [26]. However, none of these regimens has contributed to a prolonged MST of over 9 months [27]. Similarly to their reports, the MST of our patients with peritoneal metastasis, who were not treated with S-1 (historical control group), was 215 days. In contrast, the MST after initiation of S-1 administration in 16 S-1-treated patients was 550 days (18 months) with 1-year and 2-year survival rates of 53.8 and 23.1%. Moreover, 3 of our patients had long-term survival exceeding 2 years. Thus, our data showed that the MST of the S-1-treated group was significantly longer than that of the control group, and accordingly, S-1 contributed to prolonging the survival of patients with peritoneal metastasis of gastric cancer.

To our knowledge, there was only one report which compared the MST between an S-1-treated group and a historical control group in patients with peritoneal metastasis of gastric cancer [5]. However, their MST for the S-1-treated patients was only 257 days, although it was significantly longer than that of the historical control group. One explanation for this difference was that in their study, 11 of 18 patients (61%) were T4, while in our cases, 4 of 16 (25%) were T4. In fact, the MST of our T4 cases was 120 days, which is similar to their report [5].

Generally, 5-FU has various toxicities [28]. However, S-1 showed a low incidence of severe adverse reaction. Grade 3 toxicities occurred in 1 patient (leukopenia) and no grade 4 toxicities were observed. Low side effects are important to continue the chemotherapy, especially for outpatients. A frequent adverse event, that may limit continuous chemotherapy, is GI toxicity. S-1 is given in combination with Oxo to inhibit the phosphorylation of 5-FU, which causes GI toxicity, and, therefore, is expected to reduce GI toxicity [11]. In fact, in our study, no grade 3 or 4 adverse events were observed regarding GI toxicity. As a result, S-1 is feasible for long-term administration. Three of our cases were treated with S-1 for over 10 cycles, and 1 of them was treated for 27 cycles. From the viewpoint of safety, S-1 was appropriate for outpatient chemotherapy. Most of the patients were treated as outpatients, resulting in cost savings and improved quality of life.

Regarding the metastatic site, the MST of patients who had peritoneal metastasis without other distant metastases (592 days) was significantly greater than that of those with both peritoneal and other distant metastases such as of the liver or para-aortic lymph nodes (139 days). Interestingly, in the control group that was not treated with S-1, there was no significant difference in the MST between patients with and without other distant metastases. The survival of S-1-treated and nontreated patients was similar when they had both peritoneal and other distant metastases. These results suggest that S-1 has more efficacy in peritoneal metastasis rather than in other distant metastases such as to the liver and lymph nodes.

Regarding surgical treatment, the MST of patients with gastrectomy (550 days) was significantly greater than that without gastrectomy (124 days), while in the historical control group that was not treated with S-1, there was no significance between patients with and without gastrectomy. There was a possibility that the patients without gastrectomy had more advanced disease, since direct invasion of the tumor into adjacent organs such as the pancreas was the reason why gastrectomy was not performed.

In this study, we demonstrated that the MST of patients with high TP expression in primary gastric cancer was significantly shorter compared to those with low TP expression when treated with S-1. TP is known to have a high homology with platelet-derived endothelial cell growth factor, to be active as an angiogenesis-inducing factor [18], and to be related to tumor growth/progress in gastric cancers [29, 30], while it is reportedly an enzyme that converts some prodrugs to 5-FU [31]. Ichikawa et al. [32] reported that tumors with low TP expression in a primary gastric cancer had a better response than those with high TP expression, when treated with S-1. These reports support our hypothesis that tumors with low TP expression have a high sensitivity to S-1, resulting in long survival of patients with peritoneal metastasis of gastric cancer.

S-1 showed a surprisingly long-term survival with minimum toxicity and good compliance in patients with peritoneal metastasis of gastric cancer. In addition, some factors that prolong the survival of patients who had peritoneal metastasis and were treated with S-1 have been elucidated: peritoneal metastasis without other distant metastases, the combination of gastrectomy and S-1 administration, and low tumor TP expression. These results have encouraged us to conduct a large-scale study to confirm the efficacy of S-1 and establish the tumor profiling that predicts the response to S-1 in patients with peritoneal metastasis of gastric cancer.

References

▶1 Sakakura C, Hagiwara A, Nakanishi M, Shimomura K, Takagi T, Yasuoka R, Fujita Y, Abe T, Ichikawa Y, Takahashi S, Ishikawa T, Nishizuka I, Morita T, Shimada H, Okazaki Y, Hayashizaki Y, Yamagishi H: Differential gene expression profiles of gastric cancer cells established from primary tumour and malignant ascites. Br J Cancer 2002;87:1153–1161.

▶2 Kodera Y, Nakanishi H, Yamamura Y, Shimizu Y, Torii A, Hirai T, Yasui K, Morimoto T, Kato T, Kito T, Tatematsu M: Prognostic value and clinical implications of disseminated cancer cells in the peritoneal cavity detected by reverse transcriptase-polymerase chain reaction and cytology. Int J Cancer 1998;79:429–433.

▶3 Ikeguchi M, Matsumoto S, Yoshida S, Murakami D, Kanaji S, Ohro S, Yamaguchi K, Saito H, Tanabe S, Kondo A, Tsujitani S, Kaibara N: Laparoscopic-assisted intraperitoneal chemotherapy for patients with scirrhous gastric cancer. Chemotherapy 2005;51:15–20.

▶4 Kitamura Y, Hayashi K, Sasagawa T, Oguma H, Takasaki K: Pilot study of S-1 in patients with disseminated gastric cancer. Drugs Exp Clin Res 2003;29:125–130.

▶5 Osugi H, Takada N, Takemura M, Kaseno S, Lee S, Ueno M, Tanaka Y, Fukuhara K, Fujiwara Y, Kinoshita H: Oral fluoropyrimidine anticancer drug TS-1 for gastric cancer patients with peritoneal dissemination. Oncol Rep 2002;9:811–815.

▶6 Fujitani K, Tsujinaka T, Hirano M: Feasibility study of S-1 for resectable gastric cancer with peritoneal seeding. Hepatogastroenterology 2003;50:889–892.

▶7 Shirasaka T, Shimamato Y, Ohshimo H, Yamaguchi M, Kato T, Yonekura K, Fukushima M: Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 1996;7:548–557.

▶8 Shirasaka T, Shimamoto Y, Fukushima Y: Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. Cancer Res 1993;53:4004–4009.

▶9 Fukushima M, Satake H, Uchida J, Shimamoto Y, Kato T, Takechi T: Preclinical antitumor efficacy of S-1: a new oral formulation of 5-fluorouracil on human tumor xenografts. Int J Oncol 1998;13:693–698.

▶10 Kato T, Shimamoto Y, Uchida J, Ohshimo H, Abe M, Shirasaka T: Possible regulation of 5-fluorouracil-induced neuro- and oral toxicities by two biochemical modulations consisting of S-1, a new oral formulation of 5-fluorouracil. Anticancer Res 2001;21:1705–1712.

▶11 Shirasaka T, Nakano K, Takechi T, Satake H, Uchida J, Fujioka A: Antitumor activity of 1 *M* tegafur-0.4 *M* 5-chloro-2,4-dihydropyrimidine-1M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted in nude rat. Cancer Res 1996;56:2602–2606.

▶12 Aschele C, Lonardi S, Monfardini S: Thymidylate synthase expression as a predictor of clinical response to fluoropyrimidine-based chemotherapy in advanced colorectal cancer. Cancer Treat Rev 2002;28:27–47.

▶13 Terashima M, Irinoda T, Fujiwara H, Nakaya T, Takagane A, Abe K, Yonezawa H, Oyama K, Inaba T, Saito K, Takechi T, Fukushima M: Roles of thymidylate synthase and dipyrimidine dehydrogenase in tumor progression and sensitivity to 5-fluorouracil in human gastric cancer. Anticancer Res 2002;22:761–768.

▶14 Fujiwara H, Terashima M, Irinoda T, Takagane A, Abe K, Kashiwaba M, Oyama K, Takahashi M, Waesawa C, Saito K, Takechi T, Fukushima M: Quantitative measurement of thymidylate synthase and dipyrimidine dehydrogenase mRNA level in gastric cancer by real-time RT-PCR. Jpn J Cancer Res 2002;93:1342–1350.

▶15 Isshi K, Sakuyama T, Gen T, Nakamura Y, Kuroda T, Katuyama T, Maekawa Y: Predicting 5-FU sensitivity using human colorectal cancer specimens: comparison of tumor dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase activities with in vitro chemosensitivity to 5-FU. Int J Clin Oncol 2002;7:335–342.

▶16 Hirano Y, Kageyama S, Ushiyama T, Suzuki K, Fujita K: Clinical significance of thymidine phosphorylase and dihydropyrimidine dehydrogenase expression in transitional cell cancer. Cancer Chemother Pharmacol 2003;51:29–35.

▶17 Inada M, Mitsuhashi J, Sawada H, Miike N, Naoe Y, Daimen A, Koizumi K, Tsujimoto H, Fukushima M: Reduced activity of anabolizing enzymes in 5-fluorouracil-resistant human stomach cancer cells. Jpn J Cancer Res 1996;87:212–220.

▶18 Miyadera K, Sunizawa T, Haraguchi M, Yoshida H, Konstanty W, Yamada Y: Role of thymidine phosphorylase activity in the angiogenesis effect of platelet derived endothelial cell growth factor/thymidine phosphorylase. Cancer Res 1995;55:1687–1690.

19 NCI Common Toxicity Criteria. Bethesda, National Cancer Institute, Division of Cancer Treatment, 1988.

▶20 Lord RV, Salonga D, Danenberg KD, Peters JH, DeMeester TR, Park JM, Johansson J, Skinner KA, Chandrasoma P, DeMeester SR, Bremner CG, Tsai PI, Danenberg PV: Telomerase reverse transcriptase expression is increased early in the Barrett's metaplasia, dysplasia, adenocarcinoma sequence. J Gastrointest Surg 2000;4:135–142.

▶21 Farrugia DC, Ford HE, Cunningham D, Danenberg KD, Danenberg PV, Brabender J, McVicar AD, Aherne GW, Hardcastle A, McCarthy K, Jackman AL: Thymidylate synthase expression in advanced colorectal cancer predicts for response to raltitrexed. Clin Cancer Res 2003;9:792–801.

▶22 Ichikawa W, Takahashi T, Suto K, Nihei Z, Shirota Y, Shimizu M, Sasaki Y, Hirayama R: Thymidylate synthase and dihydropyrimidine dehydrogenase gene expression in relation to differentiation of gastric cancer. Int J Cancer 2004;112:967–973.

▶23 Kornmann M, Schwabe W, Sander S, Kron M, Strater J, Polat S, Kettner E, Weiser HF, Baumann W, Schramm H, Hausler P, Ott K, Behnke D, Staib L, Beger HG, Link KH: Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression levels: predictors for survival in colorectal cancer patients receiving adjuvant 5-fluorouracil. Clin Cancer Res 2003;9:4116–4124.

▶24 Ichikawa W, Takahashi T, Suto K, Yamashita T, Nihei Z, Shirota Y, Shimizu M, Sasaki Y, Hirayama R: Thymidylate synthase predictive power is overcome by irinotecan combination therapy with S-1 for gastric cancer. Br J Cancer 2004;91:1245–1250.

▶25 Funato T, Kozawa K, Fujimaki S, Miura T, Kaku M: Increased sensitivity to cisplatin in gastric cancer by antisense inhibition of the her-2/neu (c-erbB-2) gene. Chemotherapy 2001;47:297–303.

▶26 Matthew H, Kulke MD: The treatment of advanced gastric cancer: in search of the right combination. J Clin Oncol 2000;18:2645–2647.

▶27 Tahara M, Ohtsu A, Boku N, Nagashima F, Muto M, Sano Y, Yoshida M, Mera K, Hironaka S, Tajiri H, Yoshida S: Sequential methotrexate and 5-fluorouracil therapy for gastric cancer patients with peritoneal dissemination: a retrospective study. Gastric Cancer 2001;4:212–218.

▶28 Sabri B, Ceyhun C, Nezih M, Banu O, Tarkan T, Alper O, Gurhan K, Zahit B: A new perspective on cardiotoxicity of 5-fluorouracil. Chemotherapy 2004;50:113–118.

▶29 Kakeji Y, Maehara Y, Shibahara K, Hasuda S, Oshiro T, Bata H: Heterogeneity and clinical role of thymidine phosphorylase activity in gastric cancer. Oncol Rep 1999;6:1213–1216.

▶30 Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M: Thymidine phosphorylase/platelet-derived endothelial cell factor expression associated with hepatic metastasis in gastric carcinoma. Br J Cancer 1996;73:884–888.

▶31 Cook AF, Holman MJ, Kramer MJ, Trown PW: Fluorinated pyrimidine nucleosides. 3. Synthesis and antitumor activity of a series of 5'-deoxy-5-fluoropyrimidine nucleosides. J Med Chem 1979;22:1330–1335.

32 Ichikawa W, Takahashi T, Suto K, Hirayama R: Gene expressions for thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT), and thymidine phosphorylase (TP), not dihydropyrimidine dehydrogenase (DPD), influence outcome of patients (pts) treated with S-1 for gastric cancer (GC). ASCO proceedings. J Clin Oncol 2004;22:4050.

Identification of oligopeptides binding to peritoneal tumors of gastric cancer

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This is a report of *in vivo* intraperitoneal biopanning, and we successfully identified a novel peptide to target the multiple peritoneal tumors of gastric cancer. A phage display library was injected directly into the abdominal cavity of mice bearing peritoneal tumors of human gastric cancer, and phages associated with the tumors were subsequently reclaimed from isolated samples. The tumor-associated phages were amplified and the biopanning cycle was repeated five times to enrich for high affinity tumor-selective binding peptides. Finally, a tri-peptide motif, KLP, which showed homology with laminin 5 (a ligand for α3β1 integrin), was identified as a binding peptide for peritoneal tumors of gastric cancer. Phage clones displaying the sequence KLP showed 64-fold higher binding to peritoneal tumors than control phage and were preferentially distributed in tumors rather than in normal organs after intraperitoneal injection into mice. In addition, the KLP phages were more likely to bind to cancer cells in malignant ascites derived from a patient with recurrent gastric cancer. Synthesized peptide containing the motif KLP (SWKLPPS) also showed a strong binding activity to peritoneal tumors without cancer growth effect. Liposomes conjugated with SWKLPPS peptide appeared significantly more often in tumors than control liposomes after intraperitoneal injection into mice. Furthermore, modification of liposomes with SWKLPPS peptide enhanced the antitumor activity of adriamycin on gastric cancer cells. The peptide motif KLP seems a potential targeting ligand for the treatment of peritoneal metastasis of gastric cancer. (*Cancer Sci* 2006; 97: 1075–1081)

Gastric cancer is the second-most common cancer in the world. Approximately 700 000 patients a year die from gastric cancer worldwide.[1] Peritoneal metastasis is the predominant metastatic pattern in advanced gastric cancer[2,3] and the prognosis of patients with peritoneal metastasis of gastric cancer is poor. The median survival time of such patients has been reported to be 3–6 months[4] and a standard treatment for peritoneal metastasis of gastric cancer has not yet been established.[5]

A big obstacle to establishing effective therapies for peritoneal metastasis of gastric cancer is the countless localities, including invisible ones such as cancer cell clusters in malignant ascites. Therefore, the establishment of a methodology that could target individual peritoneal metastatic tumors would bring about a dramatic improvement in the therapeutic efficacy of treatments for peritoneal metastasis. Furthermore, identification of suitable ligands that associate uniquely with peritoneal tumors could enable the selective delivery of anticancer drugs to these tumors, thereby decreasing drug entry into non-target cells and potentially allowing eradication of disseminated tumor tissues.

Candidate targeting agents have been studied by several groups attempting to confer tumor tropism. The ligands that have been evaluated include a large number of antibodies, including fragments and single chain Fv molecules[6] and growth factors, such as fibroblast growth factor[7] and vascular endothelial growth factor.[8] However, this empiric approach to the identification of targeting ligands has recently been largely superseded by the use of library-based screening systems, which have been designed to allow iterative selection of high affinity ligands by repeated screening and enrichment of living libraries.[9]

In the current study, we used a phage panning technique *in vivo* to identify peptides that bind specifically to peritoneal metastatic tumors of gastric cancer. The peptide-presenting phage library used was based on a combinatorial library of random peptide heptamers fused to a minor coat protein (pIII) of the M13 phage and contains approximately 2.8×10^9 different sequences.[10] Panning with the library against peritoneal tumors *in vivo* permits the identification of binding peptide sequences by extrapolation from the corresponding DNA sequences of phages recovered from the tumor nodules.

This is a report of *in vivo* intraperitoneal biopanning, and we successfully identified peptides capable of binding to peritoneal metastatic tumors. In this strategy, phage libraries were injected directly into the abdominal cavity of mice bearing peritoneal metastatic tumors, and phages associated with the tumors were subsequently reclaimed from isolated samples. The tumor-associated phages were then amplified and the biopanning cycle was repeated five times to enrich for high affinity tumor-selective binding peptides. In addition, in order to confirm the feasibility of future applications of the identified peptides to clinical practice, the tumor-binding and anticancer activities of one of the peptides were assessed after incorporation into liposomes.

Materials and Methods

Animals. Athymic female BALB/c nu/nμ mice, 6–7 weeks of age, originated from the Central Institute for Experimental Animals (Kawasaki, Japan), and were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained in cages in a laminar airflow cabinet under specific pathogen-free conditions and provided with free access to sterile food and water.

Cell lines and cell culture. AZ-P7a cells, a human gastric carcinoma cell line, were kindly supplied by Dr T. Yasoshima (First Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan). The AZ-P7a cell line was derived from the AZ-521 human gastric cancer cell line and was previously reported to show a high potential for peritoneal metastasis in nude mice.[11]

Huh-7 cells, a human hepatocellular carcinoma cell line, were obtained from the Japan Health Science Foundation (Tokyo, Japan). DLD-1 cells, a human colorectal adenocarcinoma cell

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Abbreviations: ADM, adriamycin; BSA, bovine serum albumin; FCS, fetal calf serum; KLP, Lys-Leu-Pro; LipADM, adriamycin encapsulated in control liposome; PBS, phosphate-buffered saline; p.f.u., plaque-forming units; SWK-LipADM, adriamycin encapsulated in liposomes modified with stearoyl SWKLPPS.

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line, were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan).

All cells were maintained in RPMI-1640 medium (Sigma, St Louis, MO) supplemented with 10% FCS, 10^5 IU/L penicillin and 100 mg/L streptomycin (Sigma) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were passaged and expanded by trypsinization of the cell monolayers followed by replating every 4 days.

Mouse model of peritoneal metastasis of human gastric cancer. For mouse inoculation, cells in log-phase growth were harvested by trypsinization, and a medium containing 10% FCS was added. The cells were washed three times with PBS, resuspended in PBS, then maintained at 4°C until inoculation into mice. After fasting for 24 h, BALB/c nu/nμ mice were inoculated intraperitoneally with samples containing 1×10^7 AZ-P7a cells in 0.5 mL PBS. After 3 weeks, the inoculated mice had developed peritoneal metastases, and histological examination confirmed that these disseminated tumors consisted of AZ-P7a gastric cancer cells.

***In vivo* biopanning in mice with peritoneal metastases.** *In vivo* biopanning was carried out using the above-described mouse model of peritoneal metastasis. Three weeks after the inoculation of AZ-P7a human gastric cancer cells, the mice were anesthetized with diethyl ether and injected intraperitoneally with 2×10^{11} p.f.u. of the phage library (Ph.D.-7 M13 heptapeptide phage display peptide library kit; New England BioLabs, Beverly, MA) suspended in 1 mL of PBS. Twenty minutes after injection, the mice were killed and a few peritoneal metastatic tumor nodules were harvested from each mouse.

The harvested nodules were washed four times with PBS containing 0.5% Tween-20 (polyoxyethylene (20) sorbitan monolaurate; Kanto Chemical, Tokyo, Japan) to eliminate any unbound phages, then weighed, minced and homogenized in 5 mL PBS containing 1% protease inhibitor cocktail (Sigma) using a motor-driven Teflon-on-glass homogenizer. The homogenate was centrifuged at 450*g*. for 5 min (GS-15R; Beckman, Palo Alto, CA) and the supernatant was removed without disturbing the tissue pellet. The pellet was suspended in 5 mL of an acidic solution (0.2 M glycine-HCl, pH 2.2) for 3 min before being centrifuged at 450*g*. for 5 min to remove any weakly bound phages.[10] The remaining pellet (containing tightly bound phages) was neutralized by adding 750 μL of 1 M Tris-HCl (pH 9.1), then resuspended in 3 mL of PBS containing 0.5% Tween-20. The number of eluted phages was estimated by titering a small proportion on agar plates containing *Escherichia coli* strain ER2738 supplemented with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Wako, Osaka, Japan) and isopropyl beta-D-thiogalactopyranoside (Wako). The remaining phages were amplified by early log phase culture of ER2738 for 5 h at 37°C with vigorous shaking (150 r.p.m). The amplified phages were isolated from the resulting culture according to the manufacturer's recommended protocol, concentrated, titered and used for subsequent rounds of biopanning. In total, five consecutive rounds of biopanning were carried out in triplicate.

Isolation and sequencing of phage DNA. After each round of biopanning, individual phage clones were isolated from each replicate and their total DNA was isolated according to the recommended protocol of the sequencing kit manufacturer (Applied Biosystems, Foster, CA). The resulting DNA was used for sequencing analysis with –96 primer together with a BigDye terminator v3.0 cycle sequencing kit (Applied Biosystems). The DNA sequences were determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Searches for human proteins mimicked by the selected peptide motifs were carried out using online databases available through the National Center for Biotechnology Information website (http:http://www.ncbi.nlm.nih.gov/BLAST/).

Evaluation of the binding activities of each selected phage to peritoneal metastases of gastric cancer. After five rounds of biopanning, some phage clones were identified as showing substantial binding to peritoneal metastases. The binding activity of individual phage clones was determined as follows. AZ-P7a human gastric cancer cells were inoculated intraperitoneally into nude mice. After 3 weeks, the mice were anesthetized and injected intraperitoneally with 2×10^{11} p.f.u. of each selected phage clone suspended in 1 mL of PBS. Twenty minutes after injection, the mice were killed and a few peritoneal metastatic tumor nodules in addition to normal organs (liver, stomach and spleen) were harvested from each mouse. Samples obtained from the tumors and normal organs were weighed, washed with PBS and homogenized. Phages were quantified by titering multiple dilutions of the homogenate, as described above. A phage clone displaying no oligopeptide insert (insertless) was used as a negative control. The results were expressed as p.f.u./g tissue.

From the results of the above-described experiments, KLP-containing motifs (SWKLPPS and QPLLKLP) were selected as the most promising consensus sequences and studied in more depth.

Immunohistochemistry. Samples from tumors and normal organs were fixed in buffered formalin, embedded in paraffin, sectioned and mounted on slides. For phage immunolocalization, a rabbit anti-fd bacteriophage antibody (Sigma) was used at 1:400 dilution. Horseradish peroxidase-conjugated swine antirabbit immunoglobulins (DAKO, Carpinteria, CA) were used as the secondary antibodies at 1:50 dilution. Positive signals were revealed by the addition of diaminobenzidine tetrahydrochloride.

Measurement of binding of selected phages to human cancer cell lines *in vitro*. The binding activities of selected phage clone to AZ-P7a (human gastric carcinoma), DLD-1 (human colorectal carcinoma) and Huh-7 (human hepatocellular carcinoma) cells were determined in six-well plates.

The cells were acclimatized at 4°C for 30 min, then washed briefly with PBS before the addition to each well of 5×10^7 p.f.u. of the selected phage clone diluted into 1 mL of RPMI-1640 medium containing 1% BSA (Sigma). The phages were allowed to bind to the cells for 1 h at 4°C with gentle agitation. The media containing unbound phages were discarded, and the cells were then washed four times in PBS containing 1% BSA, before 1 mL of acidic solution (0.2 M glycine-HCl, pH 2.2) was added for 5 min. The samples were then neutralized by adding 150 μL of 1 M Tris-HCl (pH 9.1), and the cell-associated phages were recovered by lysing the cells in 1 mL/well of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA on ice for 1 h.

The recovery was determined by plaque infection assays of multiple dilutions of the eluted phages on bacterial lawns grown overnight on agar plates containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside and isopropyl beta-D-thiogalactopyranoside at 37°C.

Competitive inhibitory effects of synthesized peptides on phage accumulation *in vitro* and *in vivo*. The inhibitory effects of the synthesized peptides on phage accumulation were examined. AZ-P7a cells were preincubated with 0.1 μM, 1 μM or 10 μM of the SWKLPPS peptide or QPLLKLP peptide (synthesized by SIGMA Genosys Japan, Ishikari, Japan) for 30 min at 4°C, and then 5×10^8 p.f.u. of the selected phage diluted in 1 mL RPMI containing 1% BSA was added. The phages were allowed to bind to the cells for 1 h at 4°C with gentle agitation. Media containing unbound phages were discarded, and the cells were then washed four times for 5 min each in PBS containing 1% BSA, before the cell-associated phages were recovered by lysing the cells in 1 mL/well of 30 mM Tris-HCl (pH 8.0) containing 10 mM EDTA on ice for 1 h. The number of phages recovered was determined by titering multiple dilutions of the eluted phages as described above. The same experiment was repeated using an irrelevant heptapeptide (TTPRDAY) as a control.

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The selected phage clone (2×10^{11} p.f.u.) and 10 μM or 1 mM of each synthesized peptide were co-injected intraperitoneally into the model mice with peritoneal metastases. The mice were anesthetized and killed 20 min after injection. The peritoneal metastatic tumor nodules were harvested, weighed, washed with PBS and homogenized. The tumor-associated phages were quantified by titering multiple dilutions of the homogenate, as described above.

Evaluation of the mitogenicity of the SWKLPPS peptide in AZ-P7a cells. AZ-P7a cells were plated in 96-well plates at 5×10^3 cells/well and incubated at 37°C in RPMI medium containing 10% FCS in either the presence or absence of 1 μM, 10 μM or 100 μM of the SWKLPPS peptide. After 24, 48, 72 and 96 h, the viability of the AZ-P7a cells was assessed using the MTS assay, as described previously.[12] Media were replaced with 120 μL of FCS-free RPMI containing 20 μL of CellTiter 96 AQueous One solution reagent (Promega, Madison, WI), and the culture plates were incubated at 37°C for 2 h. Next, 100 μL of the medium was transferred to a new 96-well plate and the quantity of the formazan product present was determined by measuring the absorbance at 490 nm using a microplate autoreader (Molecular Devices, Sunnyvale, CA).

Binding of SWKLPPS-conjugated phages to floating cells in malignant ascites derived from a patient with advanced gastric cancer. A 63-year-old male patient diagnosed with advanced gastric cancer had previously been treated by total gastrectomy and systemic chemotherapy. He was admitted to Shinshu University Hospital (Matsumoto, Japan) due to anorexia and severe abdominal distension. Therefore, an abdominal paracentesis was carried out to remove the ascites as a palliative treatment for his symptoms, and gastric cancer cells were cytologically proven to be present in the ascites. A part of the ascites was used for this study. Written informed consent was obtained from the patient prior to the study.

The collected ascites were centrifuged at 250*g*. for 5 min and the supernatant was removed without disturbing the pellet. The pellet was suspended in 30 mL of PBS and centrifuged at 250*g*. for 5 min before the supernatant was removed. This procedure was then repeated. The final pellet was suspended in 30 mL PBS and transferred to a 6-well plate (3 mL/well). After acclimatization of the cells at 4°C for 20 min, 5×10^8 p.f.u. of SWKLPPS phage or insertless control phage was added to each well of the plate. The phages were allowed to bind to the cells for 30 min at 4°C with gentle agitation. Then, the fluid was collected from each well, centrifuged at 250*g*. for 5 min and the supernatant was removed. After this procedure was repeated, the pellet was suspended in 2 mL of PBS. The number of phages binding to cells was determined by titering as described above.

Accumulation of SWKLPPS-conjugated liposomes in tumors of mice with peritoneal metastases. Distearoylphosphatidylcholine (Nippon Fine Chemical, Osaka, Japan), cholesterol (Sigma) and the stearoyl 7 mer peptide SWKLPPS (molar ratio of 10:5:1) or distearoylphosphatidylcholine and cholesterol without a peptide conjugate (molar ratio of 10:5) were dissolved in chloroform, dried under reduced pressure and stored *in vacuo* for at least 1 h. Liposomes were prepared by rehydration of the thin lipid film with 0.3 M glucose then subjected to three cycles of freezing and thawing using liquid nitrogen. Next, the liposomes were sized by extruding them three times through a polycarbonate membrane filter with 100 nm pores. For a biodistribution study, a trace amount of [1α,2α(*n*)-^{3}H] cholesterol oleoyl ether (Amersham Pharmacia, Buckinghamshire, UK) was added to the initial solution.

Mice with peritoneal metastases were prepared as described above. After 2 weeks, the mice were anesthetized and injected with radiolabeled liposomes containing [1α,2α(*n*)-^{3}H] cholesterol oleoyl ether intraperitoneally. Twenty-four hours after the injection, the mice were killed under diethyl ether anesthesia. The blood was collected from the carotid artery and centrifuged (600*g* for 5 min) to obtain the plasma. After the mice had been bled, the tumors and normal organs (stomach, liver, spleen, kidney, lung and heart) were removed, washed with saline and weighed. The radioactivity in each sample was determined with a liquid scintillation counter (LSC-3100; Aloka, Tokyo, Japan). The distribution data were presented as the percentage dose/100 mg wet tissue or the percentage dose/100 μL plasma.

Evaluation of anticancer activity of ADM-encapsulated liposomes modified with SWKLPPS. ADM-encapsulated liposomes were prepared by a modification of the remote-loading method as described previously.[13] The liposomal size and composition were the same as the accumulation study of liposomes. AZ-P7a cells were plated on a 96-well plate (5×10^3 cells/well in RPMI containing 10% FCS) and cultured in a CO_2 incubator at 37°C for 24 h. Next, 20 μL LipADM or SWK-LipADM was added to each well and allowed to bind to the cells for 30 min at 37°C. Then the mediums were changed to RPMI containing 10% FCS and the cells were cultured for a further 24 h. This experiment was repeated at the ADM concentration of 0.3, 1, 3, 10, 30 and 100 mg/mL. Cell proliferation assay was carried out as follows: 10 μL of TetraColor One reagent containing tetrazolium monosodium salt (Seikagaku, Tokyo, Japan) was added to each well; cells were incubated for 3 h; and absorbance at 450 nm was measured with a reference wavelength at 630 nm in the microplate reader.

Statistics. The results are represented as the mean ± standard deviation of the data from three independent experiments. The significance of differences was evaluated using Student's *t*-test or the Mann–Whitney U-test. The level of significance was set at $P < 0.05$.

Approval for this study was obtained prior to experimentation from the ethics committee of Shinshu University, and all animal procedures were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals in Shinshu University.

Results

Iteration of consensus oligopeptide sequence binding to peritoneal metastases. Five consecutive rounds of biopanning were carried out in mice with peritoneal metastases derived from human gastric cancer cells. The phage recovery from each round increased with the number of biopanning passages, except for the third round. After five rounds of selection, 14-fold more phages were recovered from peritoneal nodules compared to using the native phage library.

After each round of biopanning, individual phage plaques were picked up. Their DNA was isolated and sequenced, and the corresponding amino acid sequences of the inserts were deduced. After the first and second rounds of biopanning, the tumor-derived sequences displayed no distinguishable homology (data not shown). However, the tumor-derived sequences from the third, fourth and fifth rounds displayed some consensus motifs, and these were selected as candidate peptides that can bind to peritoneal metastases of gastric cancer. After the fifth round of biopanning, 90–100 phage plaques were picked up from each replicate, and their DNA was sequenced. Next, we compared the relative frequencies of every tri-peptide motif in each replicate. Tri-peptide motifs with a frequency of 2.5% or more in the fifth round were selected as candidate binding peptides. The motif frequencies were calculated as the prevalence of each motif-containing peptide divided by the total number of isolated peptides. KLP was the most frequently encountered tri-peptide (3.7%), followed by Prp-Pro-Leu (PPL; 3.3%), Ile-Pro-Pro (IPP; 3.3%), Ala-Asn-Pro (ANP; 2.9%), Ser-Pro-Thr (SPT; 2.9%) and Ala-Pro-Leu (APL; 2.8%).

To determine which motif was the best binding peptide, the binding activities of selected phage clones expressing the candidate oligopeptides were assessed *in vivo* as described above. The phage

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clone expressing SWKLPPS showed the highest binding, with the recovery of 64-fold more phages compared with the insertless phage (control) (Fig. 1). Similarly, QPLLKLP showed a 43-fold higher recovery than the insertless phage. Therefore, the clones showing the best and second-best recoveries (SWKLPPS and QPLLKLP, respectively) both included the KLP motif. Accordingly, the KLP motif was selected as the most promising motif for binding to peritoneal metastases of gastric cancer.

Heptapeptides containing the consensus motif were analyzed using BLAST (National Center for Biotechnology Information) to search for similarity to known human peptides. Interestingly, KLP showed homology with laminin 5, which was reported to be a ligand for $\alpha3\beta1$ integrin.

Distribution of the selected phage in the mouse model of peritoneal metastasis. The phage clone displaying the sequence SWKLPPS was injected intraperitoneally into the model mice with peritoneal metastases. The phage accumulation in the tumors and organs was quantified by titering. The mean accumulation of the SWKLPPS phage in normal organs was less than 30% of that in tumors (Fig. 2a).

Immunohistochemistry was used to characterize the distribution of phage clones expressing the SWKLPPS peptide in the model mice with peritoneal metastases. The SWKLPPS phage showed strong binding to the tumor nodules (Fig. 2b,c), but only a low signal in normal organs such as the stomach, liver and spleen (Fig. 2e–g). Interestingly, the SWKLPPS phage appeared to be on the inside of the tumor nodules in addition to the surface, suggesting a possibility of penetration of the phage into the tumor nodules. However, the insertless phage only showed low signals in both tumors (Fig. 2d) and normal organs.

Binding activities of SWKLPPS-conjugated phages to human cancer cell lines. The binding activities of the phage clone expressing the SWKLPPS peptides was evaluated on confluent cultures of DLD-1 or Huh-7 cells, in comparison with AZ-P7a cells. The phages showed the greatest recovery from AZ-P7a cells. The recovery of the SWKLPPS phage from AZ-P7a cells was 1.3- and 13.9-fold higher than those from DLD-1 and Huh-7 cells, respectively. The similar recoveries of the SWKLPPS phage from AZ-P7a and DLD-1 can be explained by the supposition that the receptors for SWKLPPS might be similarly expressed in DLD-1 and AZ-P7a cells. However, the SWKLPPS phage bound to AZ-P7a cells 3-fold more strongly than the control phage in this *in vitro* experiment.

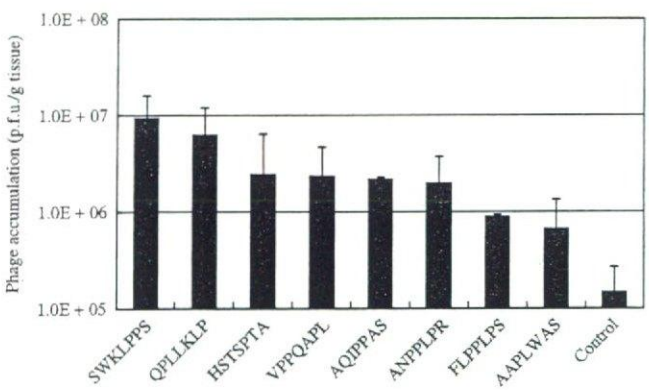

Fig. 1. *In vivo* binding activities of selected phage clones expressing the candidate peptides for binding to peritoneal tumors of gastric cancer. Each selected phage clone expressing the candidate peptides was injected intraperitoneally into model mice. The mice were killed 20 min after injection. Peritoneal tumor nodules were harvested from each mouse and homogenized, and the phages accumulated in the nodules were quantified by titering multiple dilutions of the homogenate. The results are expressed as p.f.u./g tissue, and a phage clone displaying no oligopeptide insert was used as a control.

Competitive inhibitory effects of synthesized peptides on phage accumulation *in vitro* and *in vivo*. To confirm the capacity of the synthesized peptides to accumulate in tumors, AZ-P7a cells were pre-incubated with 0.1, 1 or 10 μM of the SWKLPPS or QPLLKLP peptide before the addition of 5×10^8 p.f.u. of the selected phages. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titering the phages bound to cancer cells. It was found that pre-incubation of cells with the SWKLPPS peptide caused 66% inhibition of the binding activity of the SWKLPPS phage to these cells (Fig. 3a). In addition, the binding of SWKLPPS phage was also inhibited by the addition of QPLLKLP peptide, indicating that the KLP motif played an important role in binding to the cancer cells in both SWKLPPS and QPLLKLP.

These inhibitory effects of the SWKLPPS peptide were also confirmed in an *in vivo* experiment using the model mice with peritoneal metastases (Fig. 3b). Similar to the *in vitro* experiment, the binding of SWKLPPS phage to peritoneal tumor was inhibited by co-injection of both of SWKLPPS and QPLLKLP peptides.

Assessment of the possible mitogenicity of the selected peptide. The possibility that the SWKLPPS peptide might play a role in cancer cell growth (promotion or inhibition) was evaluated using the MTS assay. The presence of the SWKLPPS peptide had no discernible effect on cell growth (Fig. 4).

Binding of the SWKLPPS phage to floating cells in malignant ascites from a patient with gastric cancer. We carried out an *ex vivo* experiment investigating SWKLPPS phage binding to floating cells in malignant ascites from a patient with gastric cancer. The SWKLPPS phage or insertless phage was co-incubated with malignant ascites from the patient, and the number of phages bound to the cells in the ascites was examined by phage-titering. The results revealed that the SWKLPPS phage bound to cells significantly more than control phage (Fig. 5).

Tumor binding and anticancer activities of SWKLPPS-conjugated liposomes in tumors. The accumulation of SWKLPPS-conjugated liposomes in tumors of mice with peritoneal metastasis of gastric cancer after intraperitoneal injection was examined. SWKLPPS-conjugated liposomes accumulated in the tumors significantly more than control liposomes (Fig. 6). On the contrary, significantly less SWKLPPS-conjugated liposomes appeared in the liver and kidney than control liposomes. In addition, we evaluated the anticancer activity of adriamycin-encapsulated liposomes modified with SWKLPPS (SWK-LipADM), using cell proliferation assay *in vitro*. SWK-LipADM showed more efficient anticancer activity than control (LipADM) (Fig. 7).

Discussion

In the present study, we used a phage display library to identify peptide sequences capable of binding to peritoneal metastases of gastric cancer, with the aim of enabling the use of ligands for delivery of agents to such peritoneal metastases. After five rounds of selection, the consensus sequence KLP was identified and the KLP-containing peptides were examined in more depth. Sequence analysis revealed that KLP showed homology with laminin 5. Laminin 5 has been reported to serve as a high-affinity ligand for $\alpha3\beta1$ integrin.[14,15] Immunohistochemical analysis of specimens of gastric cancer resected from more than 100 patients revealed that the expression of $\alpha3\beta1$ integrin was positively correlated with the occurrence of peritoneal and liver metastases and with increased invasiveness of the tumors.[16] AZ-P7a cells, the human gastric cancer cell line used in this study, possess a high potential for peritoneal dissemination, and were reported to express a significantly higher level of $\alpha3$ integrins than AZ-521 cells, from which the AZ-P7a cell line was derived.[11] Taken together, there is a possibility that $\alpha3\beta1$ integrin is one candidate for the binding site of the KLP peptide.

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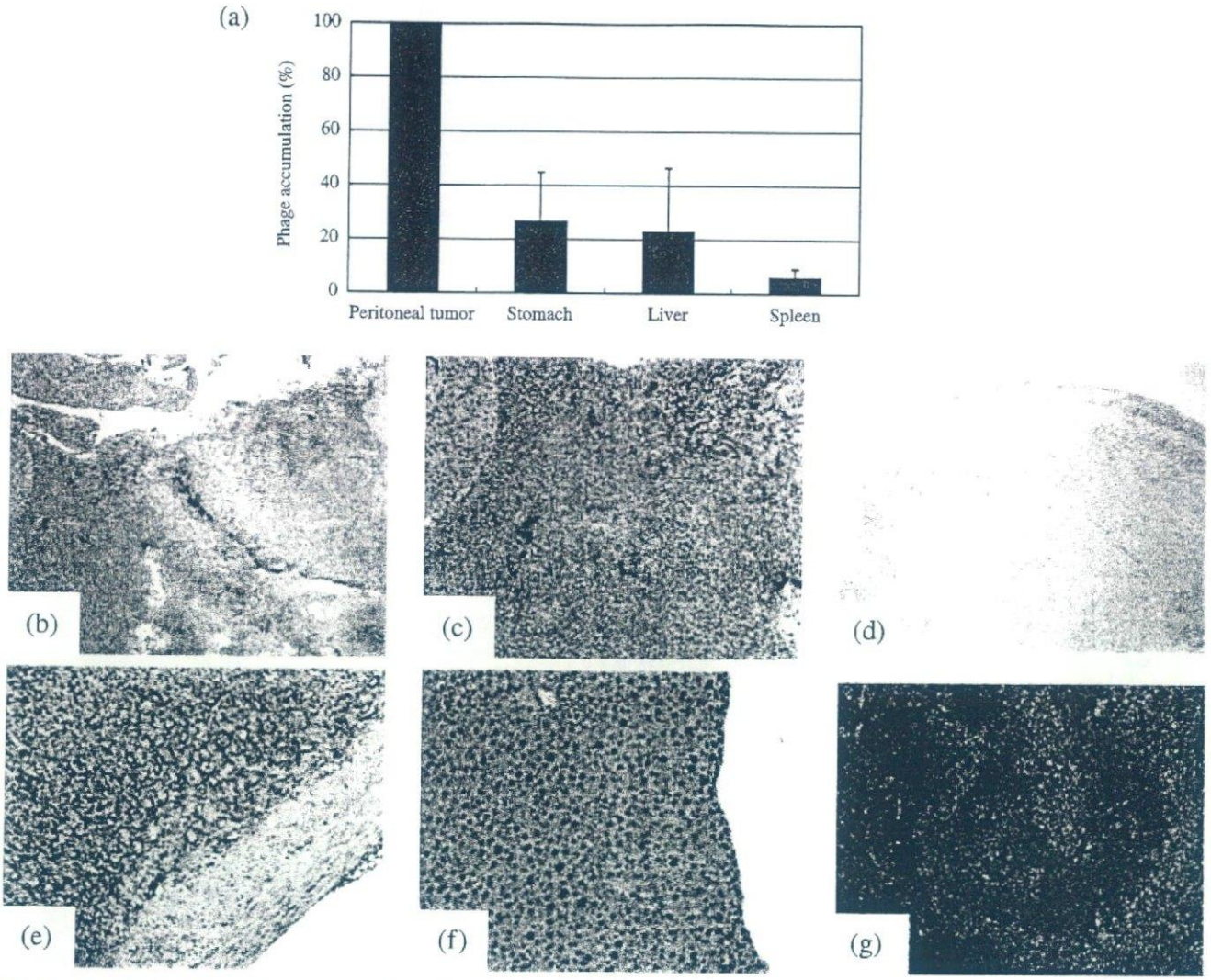

Fig. 2. Distribution of selected phage clones in model mice with peritoneal metastases. Twenty minutes after intraperitoneal injection of phage clones displaying the SWKLPPS sequence into model mice with peritoneal metastases, samples from the peritoneal tumors, normal stomach, liver and spleen were obtained. The distributions of the phages in the tumors and organs were quantified by titering and expressed as percentages of the accumulation in each organ compared to that in tumors (a). Simultaneously, the phage distributions were evaluated by immunohistochemistry (b–g). Phage accumulation is revealed by brown dots in each figure. (b, c) SWKLPPS phages in a tumor (magnification: ×40 and ×100, respectively). (d) Control phages in a tumor (magnfication: ×40). (e–g) SWKLPPS phages in the stomach, liver and spleen, respectively (magnification: ×100).

Phage display libraries have shown particular promise for elucidating receptor-binding peptides, and have recently been used *in vitro* to identify receptor-binding mimetics of fibroblast growth factor[17] and vascular endothelial growth factor.[18] However, the major strength of phage libraries is their suitability for application *in vivo* to enable the identification of ligands capable of targeting specific cells and organs.[19,20] By carrying out the selection procedure *in vivo*, the identified ligands are likely to be active under physiological conditions and their receptors will be accessible with an appropriate route of administration. In particular, this avoids the selection of ligands that bind to receptors that are inaccessible in the polarized *in vivo* cellular anatomy. Targeting systems that work well *in vitro* but fail *in vivo* due to polarization or inaccessibility of the receptors is well known.[21] Therefore, it was important that SWKLPPS was identified as a peptide that bound to peritoneal tumors in an *in vivo* experiment. Our study showed that the binding efficiency of the SWKLPPS phage to peritoneal tumors was greater *in vivo* than *in vitro* (64- *versus* 3-fold higher than the control, respectively). One possible explanation for this difference is that SWKLPPS might bind to some receptors predominantly activated *in vivo*, and works better *in vivo* than *in vitro*.

As described above, *in vivo* biopanning procedures using phage display libraries have been used to identify binding peptides for certain organs and tumors.[19–22] and in the majority of these studies, the phage libraries were injected intravenously. In contrast, intraperitoneal *in vivo* biopanning was used in our study, and we isolated the tumor-binding peptide SWKLPPS. Compared to intravenous injection, the intraperitoneal approach is clearly more useful for identifying peptides that bind to peritoneal metastases, as a larger number of phages reach the peritoneal tumors after intraperitoneal injection than after intravenous injection. Intraperitoneally injected agents, including phages, reach the tumor directly, whereas a considerable amount of intravenously injected agent is trapped by the reticuloendothelial system, such as the liver and spleen.

Another important advantage of intraperitoneal injection in animals bearing peritoneal metastases of human cancer is that the injected phages bind directly to the human cancer tissue

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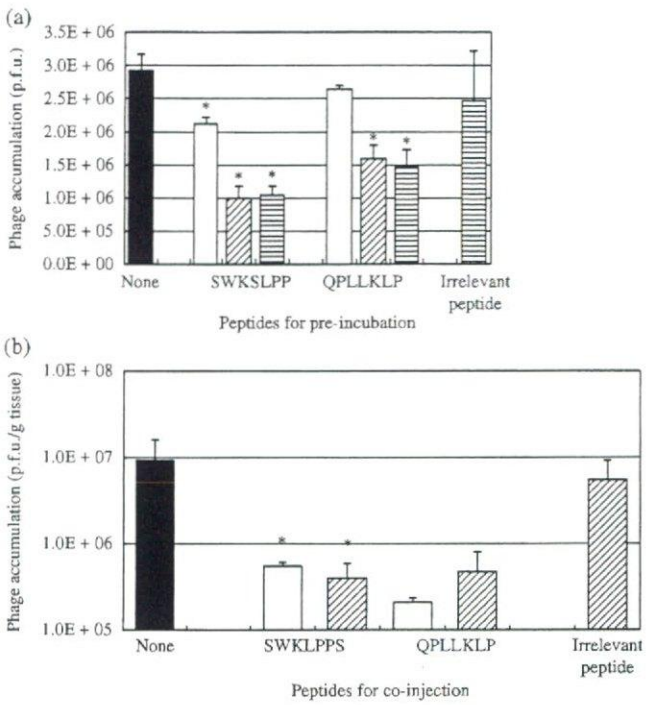

Fig. 3. Competitive inhibition of synthesized peptides against phage accumulation. AZ-P7a cells were pre-incubated with 0.1 (□), 1 (▨) or 10 μM (▤) of the SWKLPPS or QPLLKLP peptide for 30 min at 4°C, followed by the addition of 5×10^8 p.f.u. of the SWKLPPS phage *in vitro*. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titering the phages bound to the cells (a). Similarly, the SWKLPPS phage (2×10^{11} p.f.u.) and 10 μM (□) or 1 mM (▨) of each synthesized peptide were co-injected intraperitoneally into model mice with peritoneal metastases. The mice were killed 20 min after injection. Peritoneal tumor nodules were harvested, and the phages accumulated in the tumors were quantified by titering to confirm the *in vivo* inhibitory effects of the synthesized peptides on phage accumulation (b). An irrelevant heptapeptide (TTPRDAY, 10 μM *in vitro* and 1 mM *in vivo*) was used as a control. $^*P < 0.05$ compared to the control.

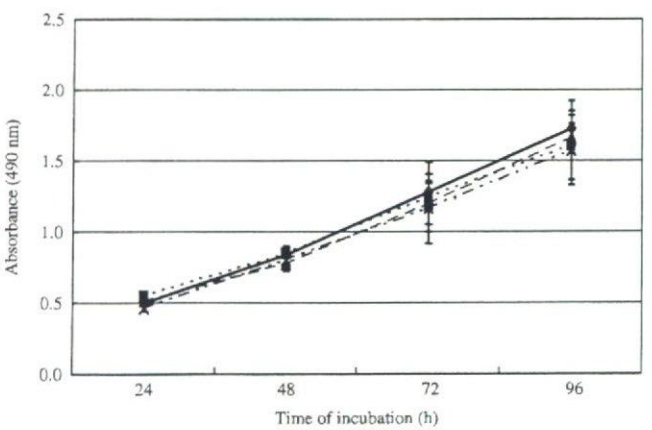

Fig. 4. Assessment of the mitogenicity of the SWKLPPS peptide in AZ-P7a cells. AZ-P7a gastric cancer cells were incubated in 96-well plates at 5×10^3 cells/well in the presence of 1 μM (■), 10 μM (▲) or 100 μM (×) of the SWKLPPS peptide or without the peptide (◆). The cell viability was monitored after 24, 48, 72 and 96 h using the MTS assay. The quantity of the formazan product present was determined by measuring the absorbance at 490 nm using a microplate autoreader.

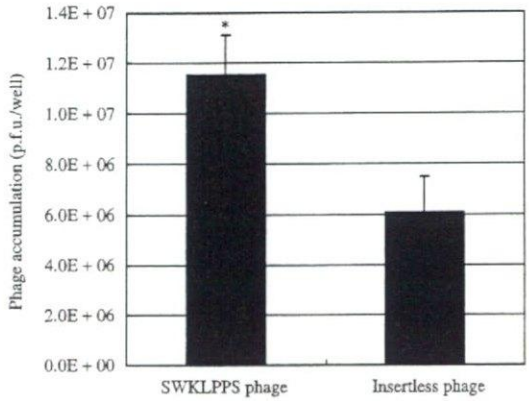

Fig. 5. Binding of the SWKLPPS phage to floating cells in malignant ascites from a patient with gastric cancer. The *ex vivo* binding activity of the SWKLPPS phage to floating cells in malignant ascites from a patient with gastric cancer was examined. The ascites from the patient was concentrated by centrifuge and co-incubated with SWKLPPS phage or insertless phage in 6-well plate. Then the number of phages binding to cells was determined by titering. $^*P < 0.05$ compared to the control.

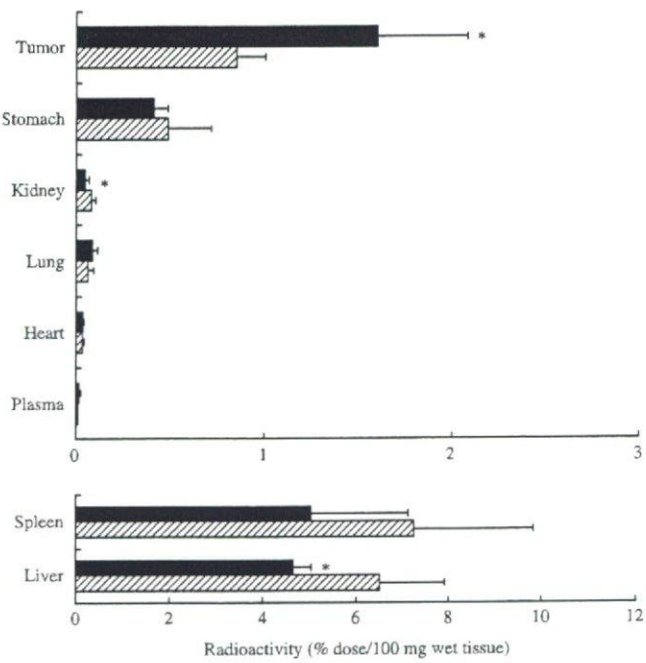

Fig. 6. Biodistribution of SWKLPPS-conjugated liposomes after intraperitoneal injection. Mice with peritoneal metastasis were anesthetized and injected with the radiolabeled liposomes containing [1á,2á(*n*)-^{3}H] cholesterol oleoyl ether with stearoyl 7 mer peptide SWKLPPS (■) or without peptide conjugates (control, ▨) intraperitoneally. The mice were killed 24 h after injection, and blood was collected and centrifuged to obtain plasma. After the mice had been bled, the tumor and normal organs were removed, washed with saline and weighed. The radioactivity in samples was determined with a liquid scintillation counter. Data are represented as the percentage of the injected dose per 100 mg wet tumor tissue or 100 μL plasma. $^*P < 0.05$ compared to the each control.

itself. Contrary to this, if the phage is injected intravenously, the majority of the phages could bind to the mouse-derived microvessels in the tumor rather than to xenografted human cancer cells. This advantage of intraperitoneal injection might have enabled the SWKLPPS phage to bind to cancer cells in ascites from a patient with carcinomatosa peritonitis, despite the fact that SWKLPPS was isolated using an animal study.

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