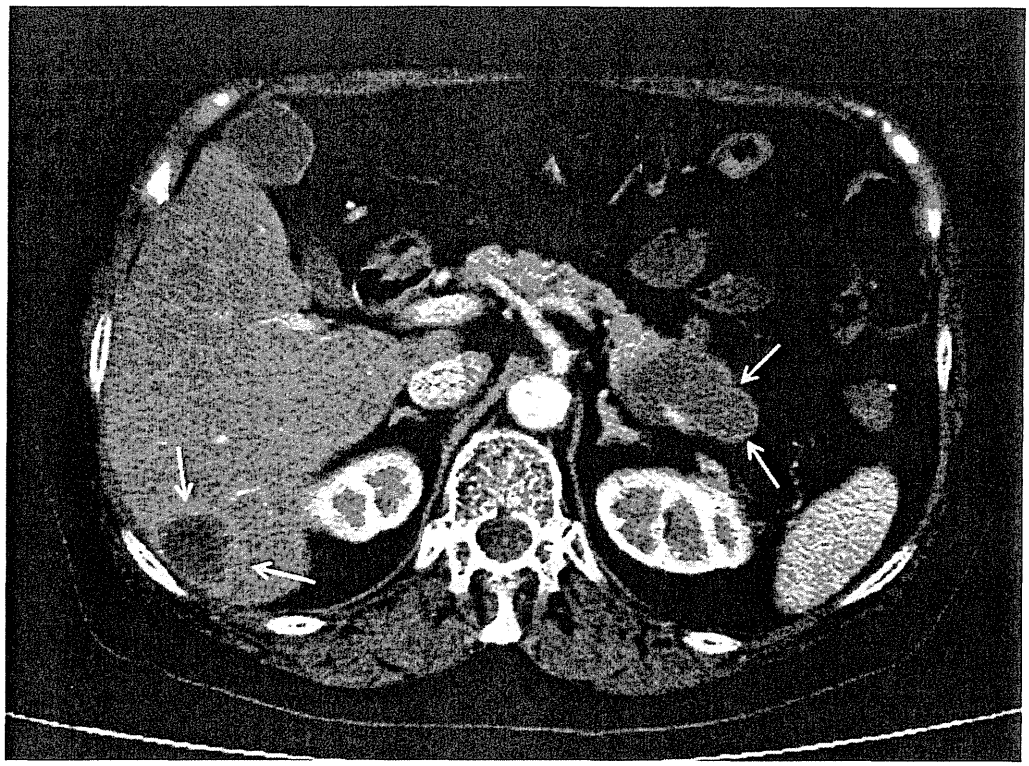

Figure 1. *Low density lesion seen in the tail of the pancreas and segment 6 of the liver, metastatic tumor from the rectal carcinoma as imaged by contrast-enhanced CT scan.*

Radiographically, no other masses were detected. At this point, it was uncertain whether the tumors in the liver and the pancreas were primary lesions or metastases from the rectal adenocarcinoma. In June 2011, the patient underwent a high anterior resection of the rectum. In a rapid diagnosis during the operation, the liver lesion was concluded to be a metastasis of the rectal adenocarcinoma, and therefore, a limited liver resection, together with resection of the pancreatic body and tail, were performed at the same time.

The rectal lesion was diagnosed histopathologically as moderately-differentiated adenocarcinoma invading into the serosal fat. The resected margins were free of tumor; however, 8 out of the 12 regional lymph nodes were positive for metastasis. The liver and pancreatic lesions showed the same morphological features in hematoxylin and eosin (H&E) staining. Immunohistological examinations revealed that the tumor cells of the rectal lesion, liver lesion and pancreas lesion were all negative for cytokeratin (CK) 7 and Mucin (MUC) 6, and all positive for CK20 and Caudal-type homeobox protein (CDX) -2. Because pancreatic metastasis of the colorectal carcinomas is rare, gene alterations of the *v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) and *tumor protein 53* (*TP53*) genes were further investigated in the rectal and pancreatic tumors. The presence of *KRAS* mutations in codons 12 and 13 were evaluated by a polymerase chain reaction (PCR)-based DNA heteroduplex assay followed by nucleotide sequencing as reported previously (10), and no *KRAS* alterations were found. The mutation hot-spots in exons 5 to 8 of the *TP53* gene were examined by direct sequencing of the PCR products, as described in a previous report (11), and the same one-nucleotide deletion followed by a stop codon (c.377del, p.Y126SfsX44) was found in both the rectal and pancreatic tumors (Table I). Taking the histopathological, immunohistochemical and genetic alteration findings into account, we considered the pancreatic tumor to be a metastasis from the rectal adenocarcinoma. As the preoperative diagnosis was a double primary cancer, we examined the chemosensitivity of both the rectal tumor and the pancreatic tumor using CD-DST to determine the most appropriate chemotherapy regimen for the patient. The results of the analysis are shown in Table II. The chemosensitivity of the metastatic pancreatic lesion was lower than that of the primary lesion for both oxaliplatin and FOLFOX (5-flurouracil, folinic acid and oxaliplatin). The pathological staging was T3 N2 M1, and based on the

Table I. *The differences in the results of the immunohistochemical and DNA mutation analyses between the primary lesion and the metastatic lesions.*

	Cytokeratin 7	MUC 6	Cytokeratin 20	CDX-2	*KRAS* mutation	*TP53* mutation
Rectum	–	–	+	+	–	c.377del, p.Y126SfsX44
Pancreas	–	–	+	+	–	c.377del, p.Y126SfsX44
Liver	–	–	+	+	Not investigated	Not investigated

MUC6: Mucin6; CDX-2: Caudal-type homeobox protein-2.

sensitivity testing, the patient underwent adjuvant chemotherapy with FOLFOX. The patient is alive and disease-free 8 months after surgery.

Discussion

The incidence of pancreatic metastases in autopsy series performed in patients with malignant neoplasms ranged from 1.6-11% (3, 4). In clinical studies among patients with solitary pancreatic masses, the frequency of pancreatic metastases ranged from 0.5 to 3% (12, 13). Renal cell carcinoma is the most common primary tumor, followed by lung cancer (adenocarcinoma and non-small cell lung carcinoma), lobular breast carcinoma, and more rarely, gastric cancer, melanoma, and soft-tissue sarcoma (2, 12, 14-17). Table III shows the details of the 30 cases with isolated metastasis to the pancreas from colorectal adenocarcinoma reported in the literature; only four cases of synchronous metastasis, including the present case, were identified out of 10 rectal adenocarcinoma cases. The treatment of colorectal cancer patients with an isolated distant organ metastasis, such as that to the brain, liver, lung, or local recurrence, by the resection of the metastases has been reported to have beneficial effects on patient survival (18-21). In patients with renal cell carcinoma, Reddy *et al.* (22) reported that the median survival after the resection of isolated pancreatic metastases was 4.8 years. However, the role of pancreatic resection for metastatic colorectal tumors is not well defined due to the paucity of such cases reported in the literature, and it is unclear whether these patients should be managed by a more conservative approach, such as chemotherapeutic management, and whether chemotherapy may offer the same results as pancreatic resection with less morbidity.

The response of recurrent disease to chemotherapeutic agents, such as 5-flurouracil, oxaliplatin and folinic acid (FOLFOX) or 5-flurouracil and folinic acid with irinotecan (FOLFIRI), has rarely been reported (14). Therefore, in the present study, we evaluated the chemotherapeutic sensitivity of cancer cells from both a primary rectal adenocarcinoma and a synchronous pancreatic metastasis using the CD-DST with multiple drug concentrations and contact durations. The

Table II. *Drug sensitivities as determined by the collagen gel droplet-embedded culture drug sensitivity test (CD-DST) in the rectal tumor and pancreatic metastasis.*

	Inhibition rate (%)	
	Primary lesion	Pancreatic metastasis
Irinotecan	36.8	27.5
Oxaliplatin	54.7	39.5
FOLFOX	63.3	53.1
FOLFIRI	42.1	41.4
5-flurouracil	30.7	41.1

FOLFOX: 5-Flurouracil+ folinic acid +oxaliplatin, FOLFIRI: 5-flurouracil+ folinic acid +irinotecan, The formula used to determine the inhibition rate is reported in the text.

CD-DST is a useful tool for the design of tailor-made chemotherapy regimens using the most suitable agents, doses, and schedules of administration (23), particularly in cases of rare tumors for which a standard chemotherapy regimen has not been established. The antitumor effect of the agents is determined by the inhibition ratio, which is calculated from the total volume of the colony that was in contact with the drug (T) and the total volume of the colony that was not in contact with the drug (C), according to the following formula: (1-T/C) ×100%. A value of more than 50% is indicative of good drug sensitivity. The primary rectal adenocarcinoma from the present patient exhibited good sensitivity to both oxaliplatin and FOLFOX, but the sensitivity to these chemotherapeutic agents was lower by more than 10% for the pancreatic metastasis. There have been no previous reports that the chemotherapy regimen was less effective for a pancreatic metastasis than for the primary colorectal carcinomalesion as determined by the CD-DST.

In conclusion, pancreatic metastases should be considerd when a patient with history of colorectal adenocarcinoma is presenting a pancreatic mass, and the present results suggest that there are potential differences in the sensitivity to chemotherapy between the primary colorectal tumor and its pancreatic metastasis.

Table III. *The nature and outcomes of pancreatic resections for colorectal metastasis: A review of the literature.*

Authors	Year	Site of primary tumor	Interval between tumors (months)	Site	Surgical procedure	Outcome	
						Dead	Alive
Present study	2012	Rectum	Synchronous	Tail	DP		7
Chao-Wei *et al.* (24)	2010	Rectum	24	Tail	DP		12
Norman *et al.* (6)	2010	Colon	108	Tail	DP	9	
Sperti *et al.* (14)	2009	Colon	48	Head	Wipple		31
		Colon	Synchronous	Head	PPPD		28
		Colon	10	Head	Wipple	17	
		Colon	36	Tail	DP		14
		Colon	24	Head	PPPD	10	
		Colon	Synchronous	Head	PPPD	15	
		Colon	Synchronous	Body	DP	5	
		Rectum	29	Tail	DP		30
		Rectum	80	Head	Enucleation	24	
Baierlein SA (25)	2008	Rectum	60	Head	PD	Not reported	
Gravalos C *et al.* (26)	2008	Colon	12	Head	PD		12
Bachmann *et al.* (27)	2007	Rectum	24	Tail	DP		1.5
		Rectum	30	Tail	DP		6
Shimoda *et al.* (28)	2007	Rectum	44	Head	PD	8	
Eidt *et al.* (29)	2007	Colon	12	Head	PPPD	105	
Matsubara *et al.* (30)	2007	Rectum	24	Head	Wipple	24	
Crippa *et al.* (31)	2006	Colon	7	Head	PPPD	13	
Torres-Villalobos *et al.* (32)	2004	Cecum	8	Tail	DP		6
Tutton *et al.* (33)	2001	Colon	23	Tail	DP		12
Pereira-Lima JC (34)	2000	Colon	36	Body	GJ	5	
Le Borgne *et al.* (17)	2000	Colon	60	Head	Wipple	12	
Yoshimi *et al.* (35)	1999	Colon	51	Tail	DP	24	
Inagaki *et al.* (36)	1998	Rectum	132	Body	DP		8
Harrison *et al.* (37)	1997	Colon	15	Head	Wipple	41	
		Colon	15	Head	Wipple	21	
Nakeeb *et al.* (38)	1995	Colon	34	Head	Wipple		43
Roland and van Heerden JA (5)	1989	Colon	Not reported	Tail	DP		27

DP: Distal pancreatectomy; GJ: gastrojejunostomy; PD: pancreaticoduodenectomy; PPPD: pylorus-preserving pancreaticoduodenectomy.

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The global histone modification pattern correlates with overall survival in metachronous liver metastasis of colorectal cancer

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Abstract. Post-translational histone modifications are known to be altered in cancer tissues, and differences in the histone modification levels have recently been used to predict the clinical outcome in patients with certain types of cancer. In this study, we evaluated the immunohistochemical staining patterns of histone H3 dimethylation and acetylation in metachronous liver metastasis of colorectal carcinomas and examined its correlation with patient prognosis. Double 2 mm core tissue microarrays were made from 54 paraffin-embedded samples of liver metastasis from colorectal adenocarcinoma, and were examined by an immunohistochemical analysis of histone H3 lysine 4 (H3K4) dimethylation, histone, H3 lysine 9 (H3K9) dimethylation and histone H3 lysine 9 (H3K9) acetylation. Positive tumor cell staining for each histone modification was used to classify patients into low- and high-staining groups, which were then examined for correlations with the clinicopathological parameters and clinical outcome. Dimethylation of H3K4 correlated with the tumor histological type (P=0.043), and acetylation of H3K9 correlated with the tumor histological type (P=0.016). In addition, lower levels of H3K4 dimethylation correlated with a poor survival rate (P=0.035). The multivariate survival analysis showed that the H3K4 dimethylation status is an independent prognostic factor for colorectal cancer patients (P=0.011). We suggest that the pattern of histone modification as detected by immunohistochemistry may be an independent prognostic factor for metachronous liver metastasis of colorectal carcinomas.

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Key words: histone modification, prognostic factor, immunohistochemistry, liver metastasis, H3K4, H3K9, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide (1). In spite of progress made in CRC chemotherapy, the outcomes of CRC with distant metastasis still remain poor. Liver metastasis of CRC is an important prognostic factor, and occurs in 20-25% of CRC patients (2). Hepatic resection is a potentially curative therapy for colorectal liver metastases. However, recurrence develops in approximately 60-70% of all such patients after hepatectomy, thus, suggesting that patients with colorectal liver metastasis often do not benefit from hepatectomy. In addition, the prognostic factors for survival that can be obtained from the resected specimens and the mechanism of tumor progression of the metastases have not yet been fully elucidated. Therefore, it is important to identify the specific biomarker of CRC outcomes, especially for patients with liver metastases.

DNA methylation and histone modification are major epigenetic mechanisms controlling gene regulation, and they are frequently altered in cancer (3). Changes in DNA methylation are closely related to patterns of histone modification (4). Cellular patterns of histone modifications have been reported as providing independent prognostic information for several cancers, including prostate (5,6), kidney (6), lung (6-8), gastric (9), ovarian (10), pancreatic (10,11), esophageal (12,13) and breast cancers (10,14). Modification of histones by methylation and acetylation at lysine residues is generally associated with gene inactivation or silencing (15-19). In CRC patients, it has also been previously reported that reduced H3 lysine 4 methylation and increased H3 lysine 9 methylation play a critical role in the maintenance of promoter DNA methylation-associated gene silencing (15). However, to date, there have been no reports on the prognostic significance of global histone modifications in cases of CRC, including liver metastasis.

In this study, we classified the expression levels of the histone dimethylation in 54 pairs of the liver metastases obtained from patients with metachronous liver metastasis of CRC. To evaluate the clinical significance of histone modification,

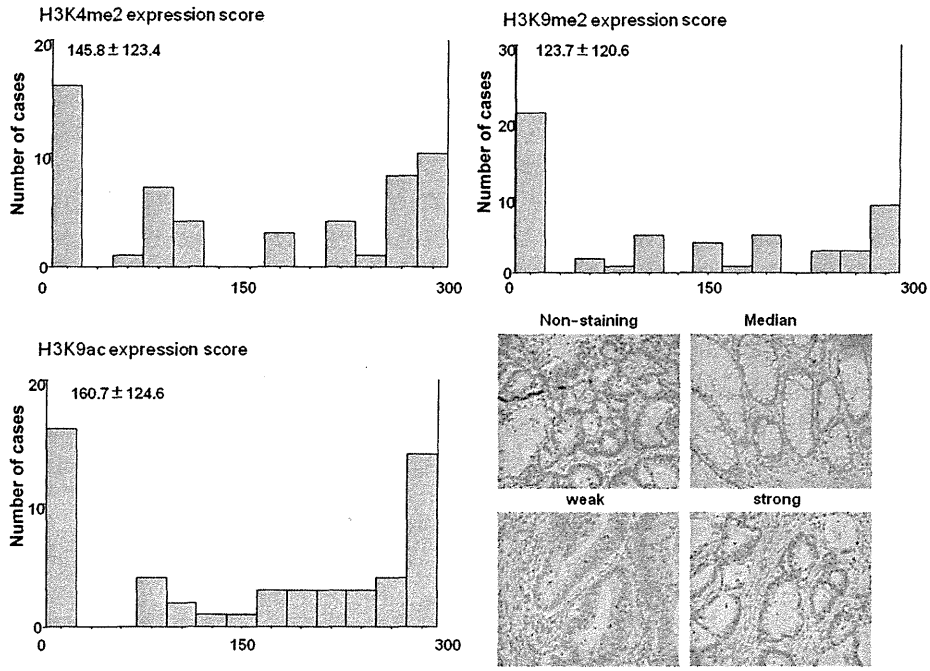

Figure 1. Histograms depicting the detection of histone modifications in CRC by immunohistochemistry. Representative examples of CRC or liver metastasis tissue cores presenting with 4 levels of staining (non-staining, weak, median and strong) of the following histone modifications: H3K4me2, H3K9me2 and H3K9ac. Original magnification, x200. Histograms showing the distribution of H-scores plotted against the number of cases for the histone modifications.

we examined the correlation between the relative expression of global histone modification patterns and the outcomes in patients with CRC.

Materials and methods

Patients and samples. We retrospectively studied the surgical specimens of liver metastasis obtained from 54 patients with metachronous liver metastasis of CRC. All of the patients had undergone curative radical (R0) resection for primary colorectal adenocarcinoma, and none of them were observed to have liver metastasis at the first operation. The metachronous liver metastases were subjected to curative radical resection at a later time. The patients underwent surgery at Kanagawa Cancer Center between January 1992 and December 2007. Primary colorectal tumors and the corresponding liver metastases were obtained from each patient. Informed consent was obtained from each patient. In all cases, archival hematoxylin and eosin-stained (H&E) slides of the respective liver metastasis specimens were retrieved and reviewed to confirm the pathological features, as well as to select suitable tissue blocks for immunohistochemical analysis. The Ethics Committees of the Kanagawa Cancer Center approved the protocol before initiation of the study. No patient had any other malignancies.

Tissue microarrays and immunohistochemistry. Microarrays consisting of cores, each 2 mm in diameter, were prepared from formalin-fixed paraffin-embedded tissue blocks of surgically removed liver metastases, and one tissue core from each liver metastasis that consisted of >80% carcinoma cells was prepared for analysis.

Immunohistochemical staining was performed using commercially-available polyclonal rabbit anti-histone antibodies raised against dimethyl-histone H3 lysine4 (H3K4me2), dimethyl-histone H3 lysine9 (H3K9me2) and acetyl-histone H3 lysine9 (H3K9ac) (Cell Signaling Technology Inc., Danvers, MA). Tissue microarray blocks were sectioned at a thickness of 4 μm and mounted on pre-coated glass slides. The sections were de-paraffinized through a graded series of xylene and rehydrated through a graded series of alcohol to distilled water. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were placed in a 95˚C solution of 0.01 M sodium citrate buffer (pH 6.0) for 40 min for antigen retrieval. Normal goat serum (5%) was then applied for 15 min to block non-specific protein binding sites. Primary rabbit anti-histone polyclonal antibodies were applied for 1 h at room temperature at the following dilutions: anti-H3K4me2 at 1:300, anti-H3K9me2 at 1:300 and anti-H3K9ac at 1:300. Immunoreactive proteins were detected using the Simple Stain MAX PO (R).

All sections were counterstained with Mayer's hematoxylin, and negative controls were included in each staining sequence. The intensity and global level of staining were scored semi-quantitatively for each tissue microarray by an investigator blinded to all of the clinicopathological variables. The global level of staining refers to the percentage of tumor cells that stained positively for an antibody within each tissue microarray at x200 magnification using a light microscope.

Scoring of immunohistochemical reactivity. Immunohistochemical scoring was done by the modified Histo-score (H-score) (20), which involves semi-quantitative assessment of both the intensity of staining (graded as 0, no staining; 1, weak; 2, median; and 3, strong, using adjacent normal mucosa as the median) and the percentage of positive cells. The range

Table I. Relationship between the expression of the histone modifications and the clinicopathological features.

Variables/categories	H3K4me2 expression			H3K9me2 expression			H3K9ac expression		
	Low (n=28)	High (n=26)	P-value	Low (n=30)	High (n=24)	P-value	Low (n=23)	High (n=31)	P-value
Age	61±9	62±9	0.892[b]	59±7	64.±11	0.075[b]	61±7	62±10	0.517[b]
Gender			0.535[c]			**0.015**[c]			0.975[c]
Male	16	17		14	19		14	19	
Female	12	9		16	5		9	12	
Size (cm)			0.554[c]			0.902[c]			0.22[c]
<5	15	16		17	14		11	20	
≥5	13	10		13	10		12	11	
Histological type[a]			**0.043**[c]			0.063[c]			**0.016**[c]
Well/Moderate	24	26		26	24		19	31	
Poor/Mucinous	4	0		4	0		4	0	
Depth of invasion			0.777[c]			0.429[c]			0.554[c]
T1-T3	14	12		13	13		10	16	
T4	14	14		17	11		13	15	
Location			0.151[c]			0.322[c]			0.724[c]
Colon	14	18		16	16		13	19	
Rectum	14	8		14	8		10	12	
Lymph node metastasis			0.171[c]			0.257[c]			0.623[c]
Absent	6	10		7	9		6	10	
Present	22	16		23	15		17	21	
Adjuvant chemotherapy			0.394[c]			0.066[c]			0.902[c]
Absent	14	10		10	14		10	14	
Present	14	16		20	10		13	17	

[a]Well, well differentiated; moderate, moderately differentiated; Poor, poorly differentiated. [b]Wilcoxon test; [c]Pearson's χ^2 test. Bold indicates values that were statistically significant (<0.05).

of possible scores is 0-300, enabling us to categorize our cases into biologically relevant groups depending on different levels of detection, which could potentially be missed using simpler scoring methods. Tumor samples with an H-score <150 for individual chromatin markers were designated as having low detection, where scores ≥150 were designated as high detection. The distribution of staining was assessed in tissue microarray sections.

Statistical analysis. The relationship between histone modification scores and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, adjuvant chemotherapy and location were evaluated with the χ^2 test and the Wilcoxon test. The postoperative survival rate and disease free survival rate were analyzed by the Kaplan-Meier method, and differences in survival rates were assessed with the log-rank test. A Cox proportional-hazard model was used for the multivariate analyses. Differences were considered significant when $P<0.05$. Each statistical analysis was performed using the SPSS II software program, version11.0.1J for Windows (SPSS, Inc., Chicago, IL).

Results

Characteristics of histone modifications. Representative immunostaining results for the three histones are shown in Fig. 1. Only nuclear staining for the three histones was regarded as positive, and cases were scored for each mark using a modified H-score. Histograms showing the staining intensity and distribution of H-scores plotted against the number of cases are shown in Fig. 1.

The expression of histone markers correlates with the clinicopathological factors. The expression scores of the histone modifications were categorized as low or high according to whether they were <150 or ≥150. The relationship between the expression levels of three histone modifications and the patient age, gender, tumor size, histological type, depth of invasion, location of lymph node metastasis and adjuvant chemotherapy after first operation, were assessed. The H3K4me2 status was positively correlated with the tumor histological type of the liver metastasis. The H3K9ac status was also positively correlated with the tumor histological type. However, the H3K9me2 status was found to only significantly correlate with gender (Table I).

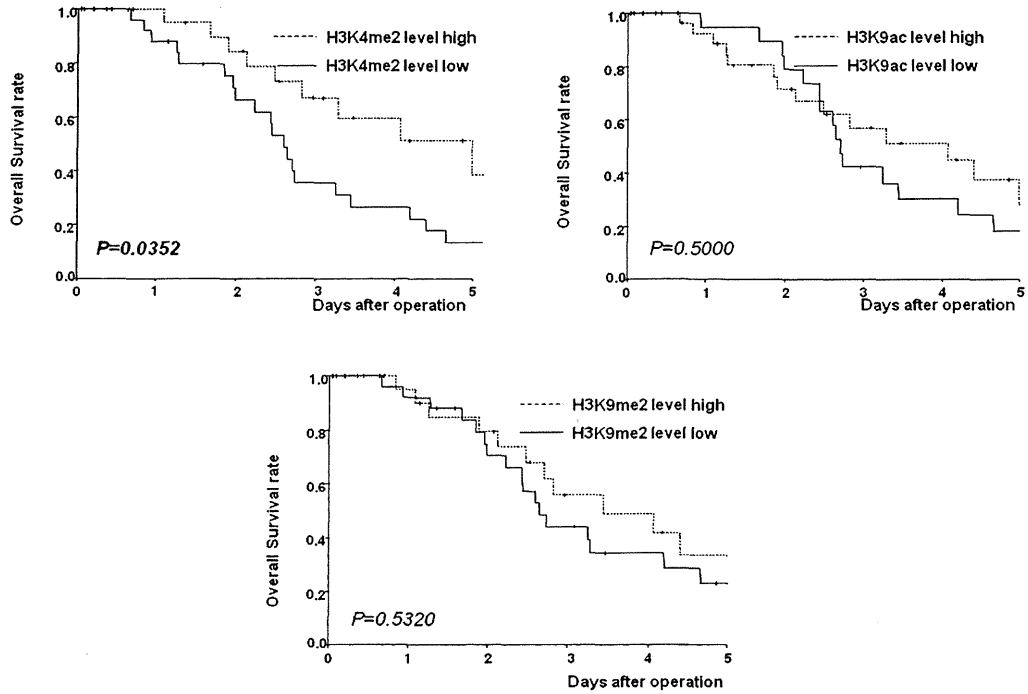

Figure 2. The Kaplan-Meier survival curves with the log-rank test for 54 patients after resection of the liver metastasis. A comparison of the overall survival based on liver metastases between the groups with high H3K4me2, H3K9me2 and H3K9ac expression and low expression, respectively. The group with high expression of H3K4me2 in liver metastases showed significantly better survival than the group with low expression (P=0.0352).

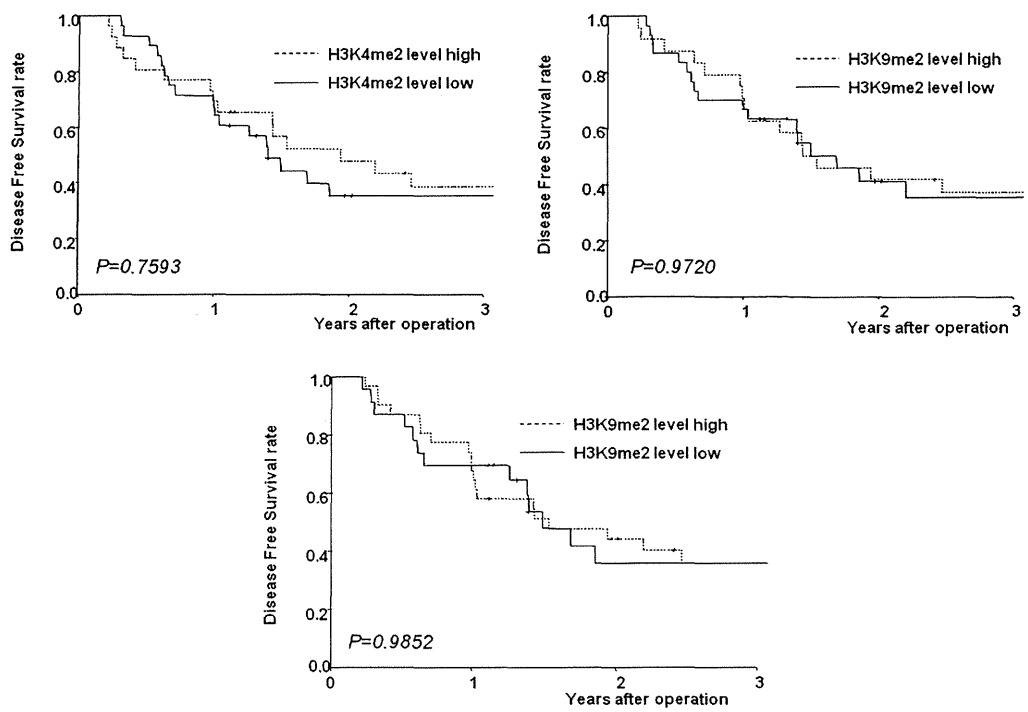

Figure 3. The Kaplan-Meier disease free survival curves with the log-rank test for 54 patients after resection of the liver metastasis. A comparison of the overall survival based on liver metastases between the groups with high H3K4me2, H3K9me2 and H3K9ac expression and low expression of each of these factors. There were no significant differences between the histone levels.

Relationships between histone markers and patient outcomes. With regard to the modification patterns, the group with high expression of H3K4me2 showed significantly better survival from the day of liver resection than those with a low expression level (P=0.0352). The group with high expression of H3K9me2 and H3K9ac showed a better survival than those with low expression, but the difference was not significant (H3K9me2, P=0.5320; H3K9ac, P=0.5000, Fig. 2). The disease free survival between the day of liver resection and the second recurrence did not significantly correlate with any

Table II. The results of a multivariate analysis of the clinicopathological factors for overall survival.

Variables/categories	n	Hazard ratio	95% CI[b]	P-value
Size (cm)				
<5	31	1		
≥5	23	1.919	0.922-3.922	0.081[c]
Histological type[a]				
Well/Moderate	50	1		
Poor/Mucinous	4	1.342	0.335-5.370	0.678[c]
Depth of invasion				
T1-T3	26	1		
T4	28	1.305	0.551-3.091	0.545[c]
Location				
Colon	32	1		
Rectum	22	1.166	0.488-2.787	0.73[c]
Lymph node metastasis				
Absent	16	1		
Present	38	0.51	0.206-1.262	0.145[c]
Preoperative CEA				
Absent	32	1		
Present	21	1.012	0.370-2.774	0.981[c]
Preoperative CA19-9				
Absent	36	1		
Present	16	2.396	1.024-5.604	**0.044**[c]
Adjuvant chemotherapy				
Absent	24	1		
Present	30	1.928	0.852-4.363	0.115[c]
H3K4me2 expression				
Low	28	1		
High	26	0.338	0.146-0.783	**0.011**[c]

[a]Well, well differentiated; Moderate, moderately differentiated; Poor, poorly differentiated. [b]CI, confidence interval. [c]Cox proportional hazard regression. Bold indicates values that were statistically significant (<0.05).

histone modification pattern (Fig. 3). The median follow-up period was 907 days.

Prognostic factors for colorectal cancer. On a multivariate Cox regression analysis including tumor size, histological type, depth of invasion, lymph node metastasis, preoperative (the first colorectal resection) CEA, CA19-9 and a lower level of H3K4me2, H3K4me2 expression and preoperative CA19-9 was an independent predictor of overall survival in patients with CRC (H3K4me2, P=0.011; CA19-9, P=0.044, Table II).

Discussion

Epigenetic alterations, such as DNA methylation and histone modification, play important roles in carcinogenesis by controlling gene activity and nuclear structural design (21,22). Recent studies have suggested that the global patterns of histone modifications can be used to predict patient outcomes for several cancers. The aim of this study was to determine the prognostic significance of histone modification in metachronous liver metastases by using an immunohistochemical analysis.

We first examined the relationship between histone modifications and clinicopathological features. In gastric carcinoma, Park *et al* (9) reported that cases with more H3K9ac-positive cells tended to be poorly differentiated adenocarcinomas. In esophageal squamous cell carcinoma, I *et al* (13) reported that the global levels of H3K9Ac and H3K9me2 in well-differentiated cases showed a tendency to be higher than those in moderately or poorly differentiated cases, but the difference in these levels were not found to be statistically significant. Our present study demonstrated that a high H3K4me2 level in the liver metastasis tended to be present in subjects with poorly differentiated adenocarcinomas, and that a positive H3K9ac status also tended to be associated with poorly differentiated adenocarcinomas.

We then examined the relationship between three histone modification levels and the outcomes of CRC with metachronous liver metastasis. Seligson *et al* (5) previously reported that prostate carcinoma patients with low cellular levels of H3K4me2 had a poorer prognosis, with a significantly increased risk of tumor recurrence compared with patients with higher levels of this modification. In lung cancer patients, a high H3K4me2 level (≥85% of tumor cells) was associated with a significantly better survival of stage I patients with large-cell or squamous cell carcinomas. In addition, low H3K9ac levels (<68% of tumor cells) were also associated with a better survival of stage I patients. In the case of pancreatic carcinoma, low cellular levels of H3K4me2 or H3K9me2 were both significant and independent predictors of poor survival in the univariate and multivariate models (11). In our study, a high level of H3K4me2 modification in liver metastases was associated with a better overall survival than a low level of this histone modification in patients with CRC. According to a univariate Cox regression analysis, a lower level of H3K4me2 modification in the liver metastases was a significant independent predictor of overall survival in these patients.

Histone modifications and DNA methylation seem to play an important role in regulating transcription and other nuclear processes. Previous reports have shown the relationship between histone modifications and DNA methylation in cancer cells. For example, promoter CpG-island hypermethylation in cancer cells has been reported to be associated with a particular combination of histone markers, for example, deacetylation of histones H3 and H4, loss of H3K4 trimethylation, and gain of H3K9 methylation and H3K27 trimethylation (23). In addition, Dnmt3L interacts with unmethylated H3K4 through its N-terminus and with Dnmt3a through its C-terminus, thus linking the DNA methylation machinery to the modification state of histone tails (24). However, while the biochemical mechanism underlying histone demethylation has been deciphered, it is still not clear how methyl groups are removed from DNA (25).

Recently, several groups have reported these epigenomic modifications to predict the clinical outcomes in human cancers, and H3K4 and H3K9 modifications are important

in the epigenetic silencing of tumor suppressor genes. Of interest, there is evidence that epigenomic profiles can predict the responses of cancer to chemotherapy, at least in pancreatic carcinoma. One report showed that the histone levels were predictive of survival specifically for patients with node-negative cancer or for those receiving adjuvant fluorouracil, but not gemcitabine (11). The impact of histone levels on CRC is not clear. If the histone modification proves to be a useful biomarker in the other cancers, then the existence of a ready-made target treatment would be invaluable for future chemotherapy.

Recent and ongoing comprehensive cancer genome studies have been identifying many gene alterations involved in histone modifications (26). Most strikingly, high-resolution SNP genotyping of medulloblastoma identified many previously unknown recurrent gene amplifications and homozygous deletions, and those events converged on genes controlling histone lysine methylation (27). We speculate that the H3K4 or H3K9 hypomethylation status may be caused by multiple genetic alterations of histone methylation modifiers, which may trigger global histone lysine modifications, rather than modification on specific gene regions of limited number, and as a whole, this is associated with the higher malignant behavior of CRC.

In conclusion, our results suggest that the pattern of histone modifications in liver metastasis as detected by immunohistochemistry can be successfully used as an independent prognostic factor for metachronous liver metastasis of colorectal cancer.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Immunohistochemical Analysis of Human Equilibrative Nucleoside Transporter-1 (hENT1) Predicts Survival in Resected Pancreatic Cancer Patients Treated with Adjuvant Gemcitabine Monotherapy

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ABSTRACT

Background. Gemcitabine is a promising adjuvant treatment for patients with resected pancreatic cancer. Human equilibrative nucleoside transporter-1 (hENT1) is the major transporter responsible for gemcitabine uptake into cells. The aim of this study was to retrospectively determine the relationship between the outcome of pancreatic cancer after surgery followed by postoperative gemcitabine monotherapy and the expression of hENT1.

Methods. A total of 27 resected pancreatic cancer patients treated with adjuvant gemcitabine were analyzed for tumor hENT1 expression via an immunohistochemical analysis. The staining intensity and the percentage of positive tumor cells were scored, and the composite score (hENT1 score) was obtained by obtaining the sum of these two scores.

Results. There were 11 patients assigned to the low hENT1 expression group, and 16 patients to the high hENT1 group. The patients with tumors that had higher hENT1 expression had a significantly longer disease-free survival (DFS) (log rank, $P = 0.022$) and overall survival (OS) ($P = 0.024$). The hENT1 expression was indicated to be a significant and independent prognostic factor for OS by the univariate ($P = 0.030$) and multivariate analyses ($P = 0.019$).

Conclusions. A high expression of hENT1 in pancreatic cancer was found to be significantly associated with a longer survival in patients who received adjuvant gemcitabine monotherapy after curative resection, and hENT1 immunohistochemistry may well serve as a significant prognostic factor for these patients.

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Pancreatic cancer remains a major therapeutic challenge. Surgery is the only potentially curative approach, and postoperative gemcitabine (2′,2′-difluorodeoxycytidine) is a promising adjuvant treatment for patients with resected pancreatic cancer.[1,2]

However, even when gemcitabine-based adjuvant therapy is administered, the prognosis of resected pancreatic cancer patients still remains poor. The median survival and the 5-year survival rate of resected patients treated with gemcitabine-based adjuvant therapy are approximately 20–23 months and 20%, respectively.[1,3–5] It is therefore important to predict the clinical outcome and select patients who are most likely to benefit from this adjuvant treatment while sparing those who are unlikely to respond from the burden of chemotherapy.

Gemcitabine is a novel pyrimidine nucleoside drug that has clinical efficiency against several common epithelial cancers.[6] Gemcitabine is transported into pancreatic cancer cells primarily by human equilibrative nucleoside transporter 1 (hENT1), then is phosphorylated to its active form, and finally it exerts its cytotoxicity.[6–8] Therefore, the expression of hENT1 in tumors is expected to be predictive for clinical outcomes in pancreatic cancer patients treated with gemcitabine.

In vitro studies have demonstrated that deficiency of hENT1 confers resistance to gemcitabine.[6] Retrospective studies in pancreatic cancer patients treated with gemcitabine also suggest an association between hENT1 expression and treatment outcome.[9,10] Recently, clinical studies of adjuvant chemoradiation therapy for resected pancreatic cancer showed that patients affected by tumors expressing high levels of hENT1 have a longer survival after gemcitabine chemotherapy than patients with a low hENT1 expression.[11,12]

These previous studies suggest that intratumoral hENT1 expression may have prognostic and/or predictive values for pancreatic cancer patients in the adjuvant setting. However, to date, clinical data from resected pancreatic cancer patients treated with adjuvant gemcitabine alone is lacking, and a question remains whether the results seen in patients who received adjuvant chemoradiation will translate to those treated with adjuvant gemcitabine monotherapy.

The aim of this study was to retrospectively determine the relationship between the outcome of pancreatic cancer patients after surgery followed by postoperative gemcitabine monotherapy and the expression level of hENT1 in the tumor as assessed by immunohistochemistry, and to clarify the prognostic values of hENT1 in these patients.

MATERIALS AND METHODS

Patients

A total of 27 pancreatic cancer patients (17 male and 10 female, aged 45–74 years), who received adjuvant gemcitabine monotherapy after curative surgical resection at Kanagawa Cancer Center between 2006 and 2008, were included in this study and were retrospectively examined for the prognostic significance of hENT1 expression as determined by an immunohistochemical analysis. Tumor staging was performed according to the International Union against Cancer (UICC) classification guidelines (2009). Informed consent was obtained from all patients to use the specimens for this study according to the institutional rules of the Kanagawa Cancer Center. The study protocol conforms to the ethical guidelines of the 1975 declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Adjuvant Treatment

Each patient received adjuvant chemotherapy using one of the following protocols: the gemcitabine biweekly protocol (gemcitabine 1,000 mg/m^2, biweekly for 6 months) or the gemcitabine standard protocol (gemcitabine 1,000 mg/m^2, days 1, 8, and 15; every 4 weeks for 6 months). Treatment was planned to start within 10 weeks postsurgery, and ten patients completed the full course of either protocol. The median cumulative total dose of gemcitabine was 10.1 g/m^2 (range, 2 to 18 g). A total of 15 patients (56%) received more than 12 g/m^2 as the cumulative total dose. The reasons for discontinuation of adjuvant chemotherapy in 17 patients included recurrent disease (15 patients, 88%) and patient withdrawal from treatment (two patients, 12%).

Immunohistochemistry

A total of 27 formalin-fixed, paraffin-embedded, pancreatic cancer sections were deparaffinized with xylene and rehydrated with a graded series of aqueous ethanol. For antigen retrieval, slides were placed in Tris/EDTA pH 9.0 buffer and autoclaved at 121°C for 15 min. Endogenous peroxidase was blocked with a 3% hydrogen peroxide solution. Then, the sections were incubated with an equilibrative nucleoside transporter 1 (ENT1) rabbit anti-human polyclonal antibody, 2.5 μg/ml (MBL International Co.), for 60 min at room temperature. Thereafter, the sections were treated with an HRP polymer kit (Polimk-2 HRP DAB Detection System for Broad, Golden Bridge International, Inc.) for signal amplification. Diaminobenzidine-hydrogen peroxide was used as the chromogen, and samples were counterstained with hematoxylin.

Determination of the hENT1 Score

The staining intensity for the hENT1 protein and the percentage of positive tumor cells was scored, and a composite score (hENT1 score) was obtained by calculating the sum of these two scores. The staining intensity for the hENT1 protein was assigned a score from 0 to 3 based on staining with 0+ thus indicating no staining; 1+, weakly positive; 2+, moderately positive; and 3+, strongly positive. The percentage of positive tumor cells was scored as follows: 0+, no positive tumor cells; 1+, <50% positive cells; 2+, 50–80% positive cells; and 3+, ≥81% positive cells. According to the hENT1 score, we classified tumors with the tumors with scores of 0–3 as having a low hENT1 expression and tumors with scores of 4–6 as having a high hENT1 expression.

Statistical Analysis

The cumulative disease-free survival rate and overall survival rate were estimated using the Kaplan–Meier method and were compared by the log-rank test. The predictors of outcome were assessed with the univariate and multivariate analyses applying the Cox proportional hazard regression model. A stepwise variable selection process

was used in the multivariate analysis to identify the most concise model for predicting cumulative survival. For all of the statistical analyses, the level of significance was set at .05. The SPSS statistical software program (SPSS for Windows 11.0J; SPSS Inc.; Chicago, IL) was used for all analyses.

RESULTS

Patients

The patient demographics and clinical characteristics are listed in Table 1. A total of 17 patients had a primary tumor located in the head of the pancreas, and ten patients had a tumor in the body to tail regions. Of the 27 patients, 26 patients (96%) had pT3 stage and 19 (70%) had pN1 disease.

Immunohistochemical Analyses

The representative results of immunohistochemical staining for hENT1 in pancreatic adenocarcinoma tissue sections are shown in Fig. 1. Immunoreactivity was observed in the cytoplasm of cancer cells. In the 27 tumor samples, ten samples (37%) showed negative staining, three samples (11.1%) showed weak staining, eight samples (29.6%) showed moderate staining, and 6 (22.2%) showed strong staining. In the 17 tumor samples that showed positive staining, two samples had 50–80% positive cells, and 15 samples had ≥81% positive cells. There were 11 patients assigned to the low hENT1 expression group and 16 patients to the high hENT expression group.

TABLE 1 Baseline patient characteristics

Age, median (range) (years)	64 (45–74)
Gender, male/female	17/10
Primary location, head/body, tail	17/10
Tumor size	
≤4.0 (cm)	14
>4.0 (cm)	13
pT stage	
T1, T2	1
T3	26
pN stage	
N0	8
N1	19
Resection status	
R0	14
R1	13
Histology	
Well to moderately differentiated	14
Poorly differentiated	11
Mucinous	2

Survival Analysis

Figure 2 shows the disease-free survival (DFS) curves and the cumulative overall survival (OS) curves for the patients who had undergone curative surgery for their pancreatic cancer, stratified by the hENT1 expression of the tumor. The OS after surgery of the patients with tumors that had a higher level of hENT1 expression was significantly longer ($P = 0.024$ by log-rank test) than that of patients whose tumors had lower hENT1 expression. There was also an improvement in the DFS after surgery for the patients with high hENT1 compared with those with low hENT1 ($P = 0.022$ by log-rank test). The median OS was 11.8 months (95% confidence interval [95% CI], 6.9–16.6) in the low hENT1 group, and 22.2 months (95% CI, 11.5–32.9) in the high hENT1 group. The median DFS was 7.3 months (95% CI, 3.6–11.1) in the low hENT1 group, and 9.3 months (95% CI, 4.2–14.5) in the high hENT1 group.

According to a univariate analysis, both hENT1 expression ($P = 0.028$) and microscopic perineural invasion ($P = 0.026$) were identified to be significant factors affecting DFS. The result of the multivariate analysis using four variables, including tumor size, hENT1 expression, microscopic vascular invasion, and microscopic perineural invasion, indicated that microscopic vascular invasion ($P = 0.003$) and microscopic perineural invasion ($P = 0.003$) were the independent prognosticators for DFS (Table 2).

The univariate analysis results indicate that hENT1 expression ($P = 0.030$) and microscopic vascular invasion ($P = 0.017$) are the significant factors affecting OS. The results of the multivariate analysis using these two variables indicated that both hENT1 expression ($P = 0.019$) and microscopic vascular invasion ($P = 0.011$) were independently and significantly associated with the OS (Table 3).

DISCUSSION

In this study, we analyzed the hENT1 protein expression using immunohistochemical analysis to determine the prognostic value of hENT1 in patients treated with adjuvant gemcitabine monotherapy after resection of pancreatic cancer. The results of the present study showed a strong relationship between hENT1 protein expression in pancreatic cancer and treatment outcomes. The patients with a high level of tumor hENT1 had a longer DFS and OS when compared with the patients with low tumor expression of hENT1. Moreover, hENT1 expression in the tumor was

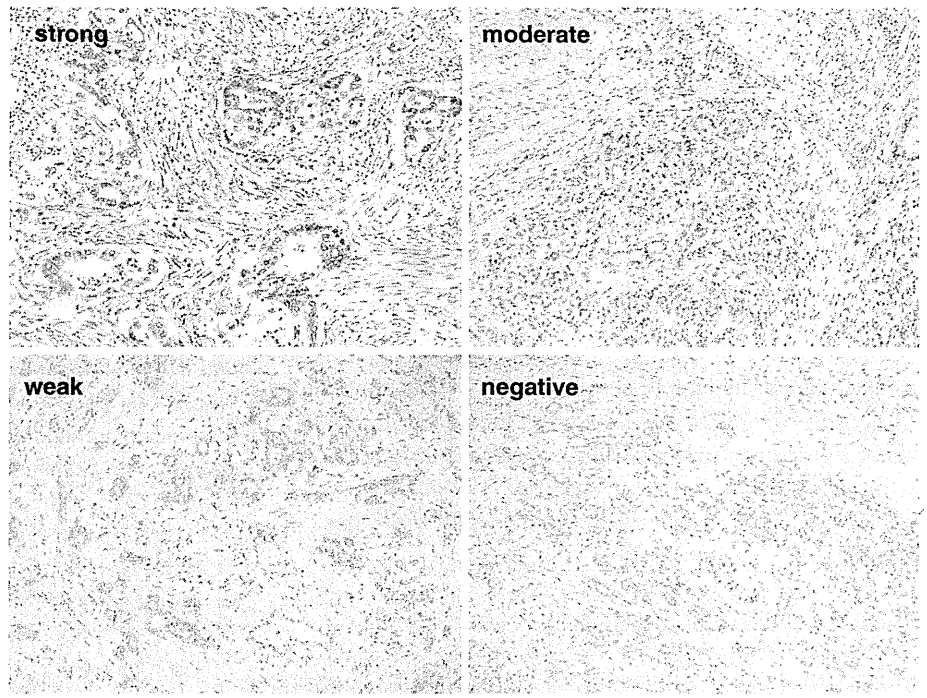

FIG. 1 Representative results of immunohistochemical staining for hENT1 in pancreatic adenocarcinoma. Immunoreactivity was observed in the cytoplasm of cancer cells

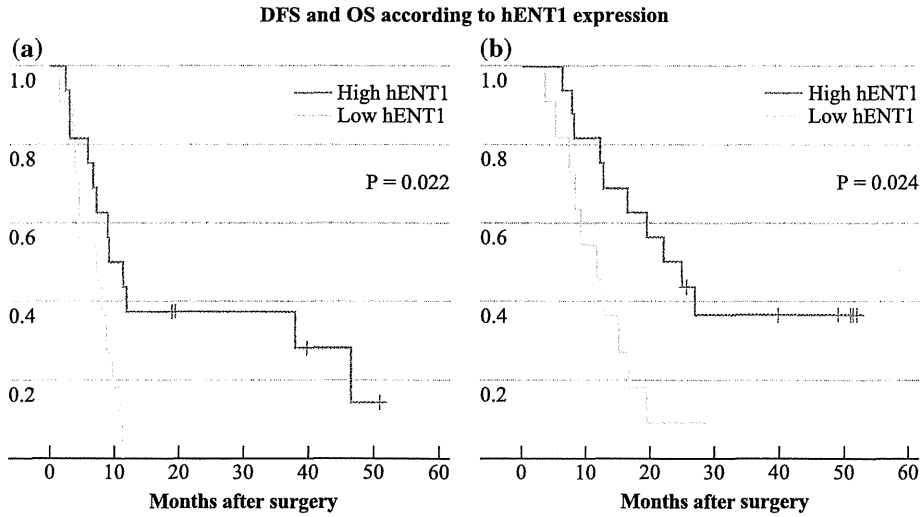

FIG. 2 The DFS and OS according to the tumor hENT expression. The DFS and OS of the patients with tumors that had a higher hENT1 expression level were significantly longer ($P = 0.022$ and $P = 0.024$, by the log-rank test) than those of the patients whose tumors had lower hENT1 expression

indicated to be a significant and independent prognostic factor for OS, along with microscopic vascular invasion, by the univariate and multivariate analyses.

Our results are in line with the results of previous reports assessing the prognostic and/or predictive value of hENT1 in pancreatic cancer.[9–12] Retrospective studies on patients with various stages of pancreatic cancer treated with gemcitabine showed pancreatic cancer with either uniformly detectable hENT1 immunostaining or a higher expression of hENT1 mRNA to have an improved clinical outcome after gemcitabine chemotherapy.[9,10]

Recently, two studies investigating the prognostic and/or predictive value of hENT1 expression in the adjuvant setting have been reported.[11,12] In the pancreatic adenocarcinoma patients treated with gemcitabine-based chemoradiation after curative resection, patients with high tumor hENT1expression had a significantly longer DFS and OS than patients with low expression when the data were adjusted for the effects of the lymph node ratio and tumor diameter.[11] In a cohort of pancreatic adenocarcinoma patients from a prospective randomized adjuvant treatment trial (RTOG9704), the hENT1 protein expression level was associated OS and DFS in pancreatic cancer patients who received gemcitabine before and after 5-FU-based chemoradiation, but not in those who received 5-FU instead of gemcitabine.[12]

Our results provide additional information regarding the prognostic and predictive value of hENT1 in the

TABLE 2 Univariate and multivariate analyses for disease-free survival

Variables	No. patients	Univariate			Multivariate		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Primary location							
Head	17						
Body, tail	10	0.686	0.279–1.686	0.411			
Tumor size							
≤4.0 (cm)	14						
>4.0 (cm)	13	2.367	0.975–5.749	0.057	0.9980	0.367–2.721	0.999
pN stage							
N0	8						
N1	19	1.376	0.561–3.376	0.485			
Resection status							
R0	14						
R1	13	1.446	0.632–3.306	0.382			
hENT1 expression							
Low	11						
High	16	0.362	0.146–0.898	0.028	0.558	0.214–1.452	0.232
Histology							
Well to moderately differentiated	14						
Poorly differentiated	11	1.105	0.475–2.572	0.816			
Mucinous	2						
Microscopic vascular invasion							
None to minimal	19						
Moderate to marked	8	2.385	0.943–6.033	0.066	5.893	1.826–19.014	0.003
Microscopic lymphatic invasion							
None to minimal	18						
Moderate to marked	9	1.746	0.734–4.148	0.207			
Microscopic perineural invasion							
None to minimal	11						
Moderate to marked	16	3.028	1.140–8.041	0.026	6.014	1.877–19.268	0.003

postoperative setting. To date, there have been two studies that have investigated the prognostic and/or predictive value of hENT1 in the postoperative setting.[11,12] However, these were studies on patients who received adjuvant chemoradiation. Therefore, the clinical data from patients who received adjuvant gemcitabine alone has been lacking, and it has remained unclear whether the results seen in patients treated with adjuvant chemoradiation would translate to those who received adjuvant gemcitabine monotherapy. Our study, in addition to emerging clinical data and the previous studies, strongly suggest that intratumoral hENT1 expression may represent a prognostic and/or predictive factor for pancreatic cancer patients who undergo surgery followed by postoperative gemcitabine-based therapy.[11,12]

The existing preclinical data also support these findings. Several studies support the idea that tumor expression of hENT1 is mechanistically and biologically relevant to the tumor resistance to gemcitabine. Because gemcitabine is hydrophilic and cannot permeate the plasma membrane by passive diffusion, it requires plasma membrane nucleoside transporter proteins to efficiently enter cells and exert its cytotoxic effect.[6–8] In vitro studies have shown that gemcitabine enters pancreatic cancer cells primarily via the hENT1 transporter, and the cells lacking hENT1 are highly resistant to gemcitabine, confirming the importance of hENT1 for the activity of gemcitabine.[6,7]

An immunohistochemical analysis of hENT1 in pancreatic cancer might thus become a useful tool for determining the appropriate use of gemcitabine and other agents such as fluorouracil plus folinic acid (5-FU/LV) in patients with resected pancreatic cancer. The results of the ESPAC-3, a phase III trial that was designed to compare the survival benefit of adjuvant fluorouracil plus folinic acid (5-FU/LV) versus gemcitabine, showed that the OS was similar in both arms.[5] An in vitro study of cultured

TABLE 3 Univariate and multivariate analyses for overall survival

Variables	No. patients	Univariate			Multivariate		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Primary location							
Head	17						
Body, tail	10	0.761	0.302–1.918	0.562			
Tumor size							
≤4.0 (cm)	14						
>4.0 (cm)	13	1.825	0.753–4.420	0.182			
pN stage							
N0	8						
N1	19	2.243	0.747–6.734	0.150			
Resection status							
R0	14						
R1	13	2.013	0.818–4.955	0.128			
hENT1 expression							
Low	11						
High	16	0.366	0.148–0.906	0.030	0.327	0.128–0.835	0.019
Histology							
Well to moderately differentiated	14						
Poorly differentiated	11	1.353	0.534–3.425	0.524			
Mucinous	2						
Microscopic vascular invasion							
None to minimal	19						
Moderate to marked	8	3.212	1.230–8.390	0.017	3.668	1.345–10.005	0.011
Microscopic lymphatic invasion							
None to minimal	18						
Moderate to marked	9	1.450	0.574–3.660	0.432			
Microscopic perineural invasion							
None to minimal	11						
Moderate to marked	16	1.607	0.638–4.052	0.314			

human mammary carcinoma cells (MDA-MB-435 s) showed that 5-FU is not a substrate of hENT1.[13] Therefore, patients with gemcitabine resistant tumors can still be treated with 5-FU based adjuvant regimens.

Of the 27 patients included in this study, 15 discontinued the adjuvant chemotherapy because of recurrent disease. This might be because most of the patients included in this study had relatively advanced stage cancer. Of the 27 patients, 26 patients (96%) had stage pT3 stage and 19 (70%) had pN1 disease. The validity of the predictive/prognostic value of hENT1 should be confirmed in larger prospective studies.

In summary, a high level of hENT1 expression in pancreatic cancer is significantly associated with a longer survival in patients who received adjuvant gemcitabine monotherapy after curative resection. Immunohistochemical analysis of the hENT1 expression may serve as a significant prognostic/predictive marker to appropriately select patients for gemcitabine-based adjuvant therapy or to select a more suitable drug for patients who have undergone resection of pancreatic cancer. However, the current study was a small scaled study, so our results warrant further investigation in larger prospective studies to confirm the predictive/prognostic value of hENT1. This report was presented in part to the 2011 Gastrointestinal Cancers Symposium of the American Society of Clinical Oncology, as part of General Poster Session B. San Francisco, California, January 2011.

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Use of omentum or falciform ligament does not decrease complications after pancreaticoduodenectomy: Nationwide survey of the Japanese Society of Pancreatic Surgery

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Background. *Wrapping is thought to prevent pancreatic fistula and postoperative hemorrhage for pancreaticoduodenectomy (PD), and we analyzed whether omentum/falciform ligament wrapping decreases postoperative complications after PD.*

Methods. *This is a retrospective study of wrapping using the omentum/falciform ligament in patients that underwent PD between January 2006 and June 2008 in 139 institutions that were members of the Japanese Society of Pancreatic Surgery.*

Results. *Ninety-one institutions responded to the questionnaires, and data were accumulated from 3,288 patients. The data from 2,597 patients were acceptable for analysis; 918 (35.3%) patients underwent wrapping and 1,679 patients did not. A pancreatic fistula occurred in 623 patients (37.3%) in the nonwrapping group, in comparison to 393 patients (42.8%) in the wrapping group (P = .006). The incidence of a grade B/C pancreatic fistula was lower in the nonwrapping group than the wrapping group (16.7% vs 21.5%; P = .002). An intra-abdominal hemorrhage occurred in 54 patients (3.2%) in the nonwrapping group, which was similar to the incidence in the wrapping group (32 patients; 3.5%). The mortality was 1.3% and 1.0% in nonwrapping and wrapping groups, respectively. A multivariate analysis revealed 7 independent risk factors for pancreatic fistula; male, hypoalbuminemia, soft pancreas, long operation time, extended resection, pylorus preservation, and omentum wrapping. There were 4 independent risk factors for early intra-abdominal hemorrhage and 2 independent risk factors for late intra-abdominal hemorrhage.*

Conclusion. *This retrospective study revealed that omentum wrapping did not decrease the incidence of pancreatic fistula. An additional validation study is necessary to evaluate the efficacy of wrapping for PD. (Surgery 2012;151:183-91.)*

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PANCREATICODUODENECTOMY (PD) is a major operation associated with a high incidence of mortality and morbidity, and numerous trials have been attempted to decrease the mortality and morbidity after PD.[1-4] The incidence of mortality has decreased at high-volume centers because of the progression of surgical techniques and perioperative treatment[5-7]; however, the incidence of morbidity still remains high.[1-4,8-10] Pancreatic fistula, delayed gastric emptying,[11,12] and postoperative hemorrhage after PD are the most frequent postoperative complications. Although delayed gastric emptying is not a lethal complication, both pancreatic fistula and postoperative intra-abdominal hemorrhage can lead to

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operation-related death.[13,14] In addition, a low incidence of complications is required in pancreatic surgery in order to administer postoperative adjuvant therapy quicker to improve the survival of patients with pancreatic cancer.[15] The International Study Group of Pancreatic Fistula (ISGPF) has proposed a consensus definition and clinical grading of postoperative pancreatic fistula, which made it possible to compare the incidence of pancreatic fistula associated with various surgical techniques.[16]

Wrapping with omentum/falciform ligament is one of the procedures to protect the surrounding organs against the pancreatic juice having autolytic activity, and this surgical technique is simple and easy for surgeons to perform. Several reports have so far shown the usefulness of wrapping after PD at individual institutions.[6,17-20] However, such wrapping may disturb the drainage of amylase-rich fluid, which might cause intra-abdominal adipose tissue inflammation like panniculitis, which could result in the occurrence of an intra-abdominal abscess.

The Japanese Society of Pancreatic Surgery (JSPS) decided to perform a nationwide survey to evaluate whether wrapping using the omentum/falciform ligament can help to prevent postoperative complications after PD.

MATERIAL AND METHODS

Patients. A nationwide survey of omental wrapping in patients who underwent PD between January 2006 and June 2008 was conducted at the initiative of JSPS to compare the patients' characteristics, preoperative status, preoperative treatment, surgical factors, perioperative status, and postoperative outcomes.

Postoperative complications. Pancreatic fistula was defined according to the ISGPF guidelines as an amylase level in the drainage fluid on postoperative day (POD) 3 that was >3 times the normal serum amylase level.[16] Postoperative intra-abdominal hemorrhage was defined as bleeding requiring a blood transfusion, reoperation, or interventional radiology. Early intra-abdominal hemorrhage indicates incomplete hemostasis and a failure of carrying out sufficient intra-operative management. It was defined as occurring within 3 days after PD, and it was not associated with any other postoperative complications. Late intra-abdominal hemorrhage is associated with other postoperative complications, including pancreatic fistula and intra-abdominal abscess. A biliary fistula was defined as the presence of bile in the drainage fluid that persisted to POD 4. An intra-abdominal abscess was defined as intra-abdominal fluid collection with positive cultures identified by ultrasonography or computed tomography associated with persistent fever and elevated white blood cells. Delayed gastric emptying is defined as output from a nasogastric tube of >500 mL per day that persists beyond POD 10, the failure to maintain oral intake by POD 14, or the reinsertion of a nasogastric tube. Vascular complications were defined as cerebral infarction, cerebral hemorrhage, and deep vascular embolization. Cardiac complications were defined as myocardial infarctions and heart failure. Respiratory complications were defined as pneumonia, pulmonary embolism, and respiratory distress requiring mechanical ventilation. Renal failure was defined as acute onset of hemodialysis. Mortality was defined as death within POD 30.

Statistical analyses. Comparisons between the 2 groups were carried out using unpaired t test for continuous data and the 2-tailed Chi-square or the Fisher exact test, where appropriate, for categorical data. The Tukey significant difference test was performed to evaluate the differences in postoperative drain amylase level among 3 groups. All factors with $P<.1$ in a univariate analysis were analyzed by a multivariate analysis. The analyses were performed with SPSS software for Windows (version 15.0; SPSS Inc., Chicago, IL). All statistical tests were 2-sided, and significance was defined as $P < .05$. The results are reported as the mean ± standard deviation.

RESULTS

Patients. Ninety-one institutions (65.5%) responded to the questionnaires, and the data from 2,597 patients were able to evaluate the occurrence of pancreatic fistula using the ISGPF criteria and postoperative hemorrhage and were acceptable for analysis in this study. The patients' characteristics are shown in Table I. The average number of PDs was 10.5 ± 11.5 and 7.5 ± 7.0 per year at the institutions with and without wrapping, respectively. There was no difference between the 2 groups ($P = .141$).

Postoperative outcome. The postoperative complications are shown in Table II. The incidence of pancreatic fistula in the wrapping group was significantly higher than that in the nonwrapping group. The intra-abdominal hemorrhagic site was identified in 24 patients in the nonwrapping group, and 22 patients (83.3%) experienced hemorrhage from an artery (9 common hepatic artery, 6 gastroduodenal artery, 4 superior mesenteric artery, 1 left gastric artery, 1 proper hepatic artery, and 1 splenic artery). The intra-abdominal hemorrhagic site was identified in 20 patients in the wrapping group, and 18 patients (90.0%) experienced hemorrhage from an artery (6 gastroduodenal artery, 5 common hepatic artery, 2 proper hepatic

Table I. Patients' characteristics

Parameter	*Nonwrapping group* (n = *1,679*)	*Wrapping group* (n = *918*)*	P *value*
Age, y (mean ± SD)	65.9 ± 10.1	66.5 ± 9.9	.100
Gender (male/female)	1,018/661	541/377	.402
Disease (carcinoma/other)	1,337/342	729/189	.895
Comorbidity			
Diabetes mellitus	466	268	.436
Respiratory disease	82	39	.463
Chronic pancreatitis	120	75	.344
Preoperative examination (mean ± SD)			
Hemoglobin (g/dL)	12.4 ± 1.7	12.4 ± 1.6	.887
Creatinine (mg/dL)	0.77 ± 0.41	0.79 ± 0.59	.490
Albumin (g/dL)	3.86 ± 0.49	3.81 ± 0.51	.017
Total bilirubin (mg/dL)	3.0 ± 4.8	2.2 ± 3.3	<.001
AST (IU/L)	70.3 ± 101.9	56.0 ± 79.3	<.001
ALT (IU/L)	100.0 ± 142.7	83.1 ± 211.6	.017
Amylase (IU/L)	123.7 ± 139.5	121.8 ± 181.6	.790
Preoperative biliary drainage	743 (44.3%)	478 (52.1%)	<.001
Duration of preoperative biliary drainage, days (mean ± SD)	25.8 ± 17.5	29.7 ± 21.4	.001
Pylorus preservation	1,016 (60.5%)	384 (41.8%)	<.001
Extended lymph node resection	1,399	773	.307
Pancreatic texture (hard/soft)	730/949	408/510	.635
Pancreaticoenterostomy			
Jejunum/stomach	1,523/156	792/126	.001
Duct-to-mucosal anastomosis	1,269 (75.6%)	778 (84.7%)	<.001
Usage of pancreatic stent tube	1,262 (75.1%)	779 (84.9%)	.001
Operative time, min (mean ± SD)	441 ± 137	534 ± 142	<.001

*Wrapping of pancreatic anastomosis or vessels, including hepatic artery, using either the omentum or falciform ligament.
ALT, Alanine aminotransferase; *AST,* aspartate aminotransferase.

artery, 1 superior mesenteric artery, 1 right hepatic artery, 1 left hepatic artery, 1 splenic artery, and 1 dorsal pancreatic artery). Thirty patients (75%) had late intra-abdominal hemorrhage accompanied by grade B + C pancreatic fistula and/or intra-abdominal abscess, and intra-abdominal hemorrhage was accompanied by all grades of pancreatic fistula in 32 patients (80%). Mortality was 1.3% and 1.0% in the nonwrapping and wrapping groups, respectively.

The level of amylase in the drainage fluid is shown in Table III. The amylase level of the omentum wrapping group was significantly lower than the other groups ($P = .027$) on POD 3.

Complications according to the material used for wrapping after PD. Two materials were used to wrap (Table IV). The incidence of grade B + C pancreatic fistula in the omentum group (23.9%) was significantly higher than in both the nonwrapping ($P < .001$) and falciform ligament groups ($P < .001$).

Complications according to the location of wrapping after PD. Wrapping was performed at 2 locations: wrapping of vessels, including the common hepatic artery, proper hepatic artery, stump of gastroduodenal artery, and portal vein, and wrapping of the pancreaticoenterostomy (Table V). The incidences of grade B + C pancreatic fistula in the anastomosis wrapping group and the vessel wrapping groups were also higher than those in the nonwrapping group.

Risk factors of postoperative complications. The risk factors of grade B + C pancreatic fistula and intra-abdominal hemorrhage were predicted using categorized data by a univariate analysis (Tables VI and VII). A multivariate analysis predicted 7 independent risk factors for grade B + C pancreatic fistula (Table VIII). A multivariate analysis revealed 4 independent risk factors for early intra-abdominal hemorrhage: male gender ($P = .017$; odds ratio [OR], 2.078), long operation time (≥600 minutes; $P = .020$; OR, 2.198), blood transfusion ($P = .002$; OR, 2.747), and soft pancreas ($P < .001$; OR, 4.184), and 2 independent risk factors for late intra-abdominal hemorrhage: male gender ($P = .017$; OR, 2.591) and soft pancreas ($P = .001$; OR, 4.274).