Intraductal carcinoma component as a favorable prognostic factor in biliary tract carcinoma

Hidenori Ojima,[1] Yae Kanai,[1,5] Motoki Iwasaki,[2] Nobuyoshi Hiraoka,[1] Kazuaki Shimada,[3] Tsuyoshi Sano,[3] Yoshihiro Sakamoto,[3] Minoru Esaki,[3] Tomoo Kosuge,[3] Michiie Sakamoto[4] and Setsuo Hirohashi[1]

[1]Pathology Division, National Cancer Center Research Institute, [2]Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, [3]Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [4]Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-0016, Japan

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The aim of this study is to evaluate the prognostic impact of an intraductal carcinoma component and bile duct resection margin status in patients with biliary tract carcinoma. An intraductal carcinoma component was defined as carcinoma within the bile duct outside the main tumor nodule consisting of a subepithelial invasive component. Surgically resected materials from 214 patients were evaluated by histological observations. Seventy-nine patients (36.9%) with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion and venous, lymphatic and perineural involvement in the main tumor nodule. An intraductal carcinoma component was inversely correlated with advanced clinical stage, and was shown to be a significantly favorable prognostic factor by both univariate and multivariate analyses. Proximal (hepatic) side bile duct resection margin status was categorized into negative for tumor cells, positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). Forty-five patients (21.0%) with an R1 resection margin had a poorer prognosis than 148 patients (69.2%) with a negative resection margin, whereas 21 patients (9.8%) with an R1 (is) resection margin did not. In patients with an R1 resection margin, the risk of anastomotic recurrence was higher, and the period until anastomotic recurrence was shorter, than in patients with an R1 (is) resection margin. Surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications. (*Cancer Sci* 2009; 100: 62–70)

Biliary tract carcinoma still has a poor prognosis, and most cases are at an advanced stage when patients present with symptoms. Previous studies of extrahepatic bile duct carcinoma and hilar cholangiocarcinoma have indicated that surgical resection is the only curative treatment for affected patients.[1–10] Biliary tract carcinoma is remarkable because of its tendency for superficial extension by wide intraductal carcinoma.[11–14] However, it is difficult to accurately estimate the extent of the intraductal carcinoma component in the biliary tract on the basis of preoperative imaging studies.[13,15–18] It is feasible that intraoperative histological diagnosis using frozen sections may detect tumor involvement at the bile duct resection margin. Surgeons are required to make an immediate decision about the resection area based on intraoperative frozen section diagnosis. However, to our knowledge, no previous study has examined the clinicopathological significance and prognostic impact of an intraductal carcinoma component with reference to bile duct resection margin status in patients with biliary tract carcinoma.

In this retrospective study, the presence or absence of an intraductal carcinoma component and bile duct resection margin status were evaluated by histological observations of all surgically resected materials from 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent. In order to provide a yardstick for surgeons who depend on the results of frozen section diagnosis during surgery, we examined the correlation between an intraductal carcinoma component and bile duct resection margin status on the one hand, and clinicopathological parameters on the other, and also the prognostic impact of an intraductal carcinoma component and bile duct resection margin status.

Materials and Methods

Patients and specimens. The study included 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent at the National Cancer Center Hospital, Tokyo, Japan, between May 1965 and December 2003. Patients who died in hospital or within 100 days after surgery, and patients who underwent biopsy or palliative surgery, were not included. The included patients comprised 150 men and 64 women, ranging in age from 33 to 83 (mean 63.4) years.

The main tumor nodule was located in the lower, middle and upper thirds of the extrahepatic bile duct, the entire extrahepatic bile duct, the hilar bile duct, and intrahepatic bile duct adjacent to the hilar area in 27, 38, 14, 5, 77, and 53 patients, respectively. Patients with carcinoma of the peripheral intrahepatic bile duct were excluded. Pancreatoduodectomy (PD), extrahepatic bile duct resection (EHBD), hepatic resection with extrahepatic bile duct resection (HR+EHBD), hepatic resection (HR) and combined hepatectomy and pancreatoduodectomy (HPD) were performed in 47, 19, 124, 16 and 8 patients, respectively. The formalin-fixed surgically resected specimens were cut into slices at intervals of 0.5–0.7 cm, and all the sections were embedded in paraffin and routinely processed for microscopical examination. All tumors were classified according to the pathological tumor-node-metastasis (TNM) classification.[19] Intrahepatic bile duct carcinomas adjacent to the hilar area, for which TNM criteria have never been established, were classified according to the TNM classification for extrahepatic bile duct carcinoma. This study was approved by the Ethical Committee of the National Cancer Center, Tokyo.

Evaluation of an intraductal carcinoma component and bile duct resection margin status. The intraductal carcinoma component was defined as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component (Fig. 1). For cases in which intraoperative frozen section diagnosis of the ductal stump had been performed, the proximal (hepatic) side bile duct resection margin status was histologically assessed by review of the frozen section and its re-fixed permanent section with reference

[5]To whom correspondence should be addressed. E-mail: ykanai@ncc.go.jp

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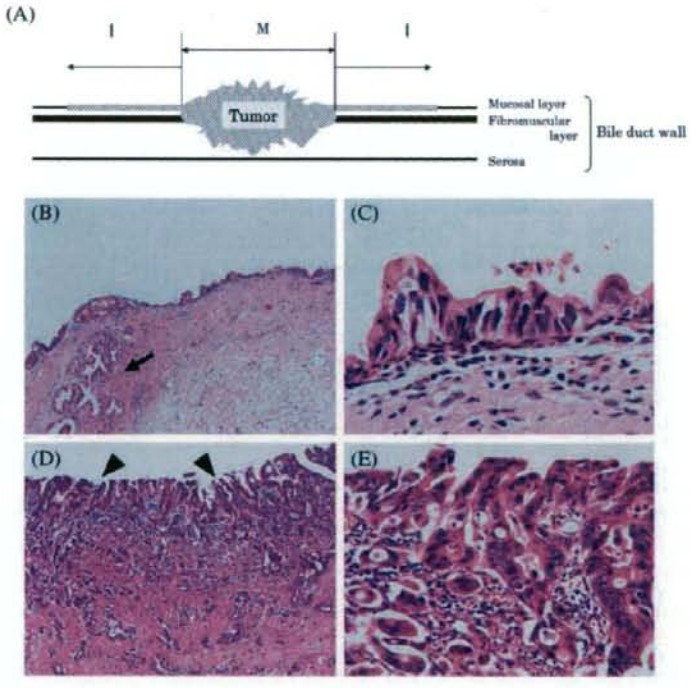

Fig. 1. Definition of an intraductal carcinoma component (I). (A) I is defined as intraductal carcinoma in the bile duct and its small branch outside the main tumor nodule (M) consisting of a submucosal invasive component. (B and C) Microscopic view of an example of I in the bile duct and its small branch (arrow). Hematoxylin and eosin (H&E) stain, original magnification ×40 (B) and ×400 (C). (D and E) Microscopic view of an example of M. Carcinoma *in situ* inside M (arrow heads) is not considered as I in this study. H&E stain, original magnification ×40 (D) and ×200 (E).

to the extent of the tumor in formalin-fixed surgically resected specimens. For cases in which intraoperative frozen section diagnosis of the ductal stump had not been performed, proximal side bile ductal resection margin status was histologically assessed by review of the formalin-fixed surgically resected specimens.

Follow-up and assessment of anastomotic recurrence at the bile duct resection margin. All 214 patients were followed for more than 100 days, and the mean duration of follow-up was 1215 days. Follow-up examination was performed using computed tomography, abdominal ultrasonography, and measurement of the serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels every 3–6 months by surgeons. Anastomotic recurrence at the proximal side of the bile duct resection margin was diagnosed only in patients with a positive resection margin. In such patients, when a mass lesion was detected after dilatation of the bile duct in the residual liver because of obstruction of the anastomosis site, using radiological evaluation including computed tomography and ultrasonography, surgeons considered that the patient had anastomotic recurrence (not local recurrence in which perineural invasion around the hepatic artery and/or involved regional nodes first formed a mass lesion). Causes of death were determined from the medical records.

Statistical analyses. Correlations between presence or absence of an intraductal carcinoma component and bile duct resection margin status on the one hand and clinicopathological parameters on the other were analyzed using chi-squared test.

Person-days of follow-up were calculated from the date of surgical resection until date of death or end of the study period (March 8, 2005), whichever occurred first. The crude rate of all-cause deaths was calculated by dividing the number of deaths by the number of person-days. Similarly, person-days of follow-up were calculated from the date of surgical resection until date of death, date of diagnosis of anastomotic recurrence, or end of the study period (March 8, 2005), whichever occurred first. The crude rate of recurrence at the proximal side bile duct resection margin was calculated by dividing the number of cases with recurrence by the number of person-days. Survival curves were constructed using the Kaplan–Meier method, and differences in survival were evaluated using the log-rank test. The Cox proportional hazards model was used to estimate hazard ratio (HR) and 95% confidence interval (CI) of death or anastomotic recurrence by clinicopathologic factors using the SAS program (PROC PHREG) (SAS Institute Inc., Cary NC, US). All tests were two-sided and differences at $P < 0.05$ were considered statistically significant.

Results

Univariate analysis of correlation between an intraductal carcinoma component and clinicopathological parameters. An intraductal carcinoma component was positive in 79 (36.9%) of the 214 examined patients. Correlations between an intraductal carcinoma component and clinicopathological parameters were examined using univariate analysis (Table 1). Location of the main tumor nodule ($P = 0.007$), and histologic type ($P < 0.0001$) were significantly correlated with an intraductal carcinoma component (Table 1). Tumor size ($P = 0.01$), depth of invasion ($P < 0.0001$), venous involvement ($P < 0.0001$), lymphatic involvement ($P = 0.0006$), perineural involvement ($P < 0.0001$), the pathological assessment of the primary tumor (pT) ($P < 0.0001$), and pathological TNM stage ($P < 0.0001$) were each inversely correlated with presence of an intraductal carcinoma component: patients with an intraductal carcinoma component infrequently developed large tumors, infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule, and were infrequently at an advanced stage when diagnosed (Table 1).

Table 1. Correlation between an intraductal carcinoma component and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases		*P* for difference*
	Intraductal carcinoma component		
	Negative (n = 135)	Positive (n = 79)	
Age (years)			0.01
<65	73	29	
≥65	62	50	
Sex			0.85
Male	94	56	
Female	41	23	
Location of the main tumor nodule			0.007
Lower third of extrahepatic bile duct	15	12	
Middle third of extrahepatic bile duct	17	21	
Upper third of extrahepatic bile duct	6	8	
Entire extrahepatic bile duct	2	3	
Hilar bile duct	54	23	
Intrahepatic bile duct	41	12	
Histologic type			<0.0001
Adenocarcinoma	129	55	
Papillary adenocarcinoma	1	21	
Others	5	3	
Tumor size (cm)			0.13
<3	54	40	
≥3	81	39	
Differentiation of adenocarcinoma			0.50
Well	34	18	
Moderate	80	29	
Poor	15	8	
Depth of invasion			<0.0001
Carcinoma *in situ* or invasion to fibromuscular layer	1	16	
Invasion into subserosa or beyond bile duct wall	134	63	
Venous involvement			<0.0001
Absent	6	19	
Present	129	60	
Lymphatic involvement			0.0006
Absent	9	18	
Present	126	61	
Perineural involvement			<0.0001
Absent	10	24	
Present	125	55	
pT classification			<0.0001
pT1-2	11	40	
pT3-4	124	39	
pN classification			0.06
pN0	64	48	
pN1	71	31	
TNM stage			<0.0001
0, IA, IB	8	32	
IIA	53	14	
IIB	62	28	
III	12	5	

*Chi-squared test.

Univariate analysis of correlation between an intraductal carcinoma component or clinicopathological parameters on the one hand and prognosis of patients on the other. Overall survival rates after resection were 33.2% at 5 years and 22.9% at 10 years. Hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and other clinicopathological parameters were examined using univariate analysis (Table 2). Patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 2).

Multivariate analysis of prognostic impact of an intraductal carcinoma component. When all 214 patients were examined by multivariate analysis adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement and TNM stage, patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 3). When only the 117 patients who underwent complete resection (proximal side bile duct resection margin for all

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Table 2. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate†	Crude HR	95% CI	*P* for trend
Intraductal carcinoma component						
Negative	96	136 804	70.2	1.00		
Positive	35	123 209	28.4	0.39	0.27, 0.58	
Age (years)						
<65	58	137 562	42.2	1.00		
≥65	73	122 451	59.6	1.33	0.94, 1.87	
Sex						
Male	94	179745	52.3	1.00		
Female	37	80 268	46.1	0.84	0.57, 1.23	
Location of the main tumor nodule						
Lower third of extrahepatic bile duct	17	43 899	38.7	1.00		
Middle third of extrahepatic bile duct	23	43 696	52.6	1.04	0.56, 1.96	
Upper third of extrahepatic bile duct	10	18 228	54.9	1.17	0.54, 2.56	
Entire of extrahepatic bile duct	3	4837	62.0	1.07	0.31, 3.67	
Hilar bile duct	46	87 502	52.6	1.09	0.63, 1.91	
Intrahepatic bile duct	32	61 851	51.7	1.14	0.63, 2.06	
Histologic type						
Adenocarcinoma	123	211 330	58.2	1.00		
Papillary adenocarcinoma	5	37 000	13.5	0.25	0.10, 0.62	
Others	3	11 683	25.7	0.51	0.16, 1.61	
Tumor size (cm)						
<3	46	134 392	34.2	1.00		
≥3	85	125 621	67.7	1.82	1.27, 2.61	
Differentiation of adenocarcinoma						
Well	36	70 278	51.2	1.00		
Moderately	67	126 210	53.1	1.13	0.75, 1.69	
Poorly	20	14 842	134.8	2.56	1.47, 4.44	
Depth of invasion						
Carcinoma *in situ* or invasion to fibromuscular layer	3	37 435	8.0	1.00		
Invasion into subserosa or beyond bile duct wall	128	222 578	57.5	6.44	2.04, 20.3	
Venous involvement						
Absent	6	63 305	9.5	1.00		
Present	125	196 708	63.5	5.80	2.54, 13.3	
Lymphatic involvement						
Absent	9	76 294	11.8	1.00		
Present	122	183 719	66.4	4.67	2.25, 9.67	
Perineural involvement						
Absent	11	72 565	15.2	1.00		
Present	120	187 448	64.0	3.67	1.95, 6.89	
pT classification						
pT1-2	23	89 367	25.7	1.00		
pT3-4	108	170 646	63.3	2.32	1.47, 3.66	
pN classification						
pN0	57	176 738	32.3	1.00		
pN1	74	83 275	88.9	2.56	1.80, 3.65	
TNM stage						
0,IA,IB	15	77 359	19.4	1.00		<0.01
IIA	39	91 428	42.7	2.26	1.24, 4.12	
IIB	65	75 858	85.7	4.21	2.37, 7.46	
III	12	15 368	78.1	3.80	1.77, 8.15	

†per 100 000 person-days.

patients, distal [duodenal] side bile duct resection margin for patients who underwent HR + EHBR, resected margin of the pancreas for patients who underwent PD were all negative) were examined in order to eliminate the effect of surgical curability, an intraductal carcinoma component was still a favorable prognostic factor (Table 3).

Correlation between an intraductal carcinoma component and bile duct resection margin status. Although an intraductal carcinoma component has been proven to be a favorable prognostic factor, it is feasible that patients with such components frequently have tumor involvement at the bile duct resection margin. Therefore, the correlation between an intraductal carcinoma component and proximal side bile duct resection margin status (negative or positive) was examined statistically (Table 4). An intraductal carcinoma component was found to be correlated with bile duct resection margin status: patients with an intraductal carcinoma component more frequently had a positive resection margin than patients without such a component ($P = 0.0192$, Table 4). In

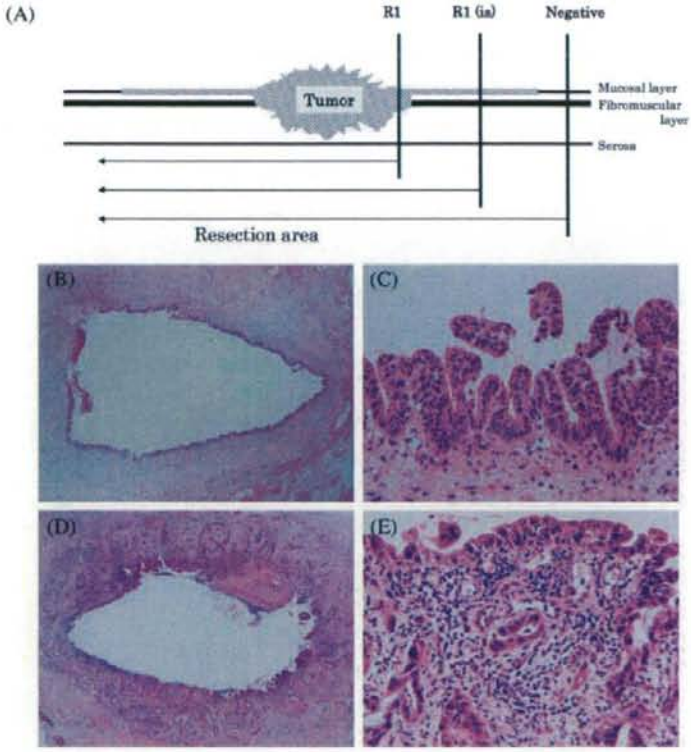

Fig. 2. Definition of bile duct resection margin status. (A) Bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). (B and C) Microscopic view of an example of an R1 (is) bile duct resection margin. Hematoxylin and eosin (H&E) stain, original magnification ×12.5 (B) and ×200 (C). (D and E) Microscopic view of an example of an R1 bile duct resection margin. H&E stain, original magnification ×12.5 (D) and × 200 (E).

Table 3. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by an intraductal carcinoma component

	Adjusted HR	95% CI
Total (214 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.50	0.31, 0.79
Complete resection cases (117 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.31	0.13, 0.73

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement, and TNM stage.

Table 4. Correlation between an intraductal carcinoma component and proximal side bile duct resection margin status

	No. of cases		*P* for difference*
	Bile duct resection margin status		
	Negative ($n = 148$)	Positive ($n = 66$)	
Intraductal carcinoma component			0.0192
Negative	101	34	
Positive	47	32	

*Chi-squared test.

order to further examine the clinicopathological significance and prognostic impact of bile duct resection margin status with reference to an intraductal carcinoma component, proximal side bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], or positive with a subepithelial invasive component (R1) (Fig. 2). According to the International Union Against Cancer, when invasive carcinoma is completely resected but histology shows an *in situ* component at the resection margin, the residual tumor is defined as R1 (is).[20] When the surgeon considers that resection has been complete but histology shows invasive carcinoma at the resection margin, the residual tumor is defined as R1.[20]

Univariate analysis of correlations between bile duct resection margin status and clinicopathological parameters. Bile duct resection margin status was negative, R1 (is) and R1 in 148 (69.2%), 21 (9.8%) and 45 (21.0%) of the 214 examined patients, respectively. Correlations between bile duct resection margin status and clinicopathological parameters were examined by univariate analysis (Table 5). Location of the main tumor nodule ($P = 0.0004$), histological type ($P = 0.008$) and venous involvement ($P = 0.009$) were each significantly correlated with bile duct resection margin status (Table 5).

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Table 5. Correlation between bile duct resection margin status and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases			*P* for difference*
	Proximal side ductal resection margin			
	Negative ($n = 148$)	R1 (is) ($n = 21$)	R1 ($n = 45$)	
Age (years)				0.09
< 65	77	6	19	
≥ 65	71	15	26	
Sex				0.81
Male	103	16	31	
Female	45	5	14	
Location of the main tumor nodule				0.004
Lower third of extrahepatic bile duct	23	2	2	
Middle third of extrahepatic bile duct	25	8	5	
Upper third of extrahepatic bile duct	8	1	5	
Entire of extrahepatic bile duct	0	1	4	
Hilar bile duct	53	7	17	
Intrahepatic bile duct	39	2	12	
Histologic type				0.008
Adenocarcinoma	129	13	42	
Papillary adenocarcinoma	15	6	1	
Others	4	2	2	
Tumor size (cm)				0.34
< 3	65	12	17	
≥ 3	83	9	28	
Differentiation of adenocarcinoma				0.16
Well	33	7	12	
Moderately	78	4	27	
Poorly	18	2	3	
Depth of invasion				0.06
Carcinoma *in situ* or invasion to fibromuscular layer	14	3	0	
Invasion into subserosa or beyond bile duct wall	134	18	45	
Venous involvement				0.009
Absent	20	5	0	
Present	128	16	45	
Lymphatic involvement				0.18
Absent	22	3	2	
Present	126	18	43	
Perineural involvement				0.57
Absent	26	3	5	
Present	122	18	40	
pT classification				0.08
pT1-2	37	8	6	
pT3-4	111	13	39	
pN classification				0.48
pN0	80	12	20	
pN1	68	9	25	
TNM stage				0.35
0, IA, IB	28	7	5	
IIA	48	5	14	
IIB	59	9	22	
III	13	0	4	

*Chi-squared test.

Univariate and multivariate analysis of prognostic impact of bile duct resection margin status. Univariate analysis revealed that although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 6). Surgical resection procedure, which was not examined in Table 3, is addressed in Table 6. None of the 66 patients with a positive resection margin [both R1 (is) and R1] had received any adjuvant therapy until recurrence was diagnosed.

When adjusted for age, operation day, surgical resection procedure, tumor size, histologic type, tumor differentiation, depth of invasion and venous involvement, although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 7).

Table 6. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by bile duct resection margin status and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate*	Crude HR	95% CI	*P* for trend*
Bile duct resection margin status						
Negative	82	209 492	39.1	1.00		<0.01
R1 (is)	11	22 476	48.9	1.00	0.53, 1.88	
R1	38	28 045	135.5	2.80	1.88, 4.18	
Surgical resection procedure						
PD	29	62 430	46.5	1.00		
EHBR	14	26 842	52.2	1.03	0.54, 1.94	
HR+EHBR	75	129 281	58.0	1.08	0.70, 1.67	
HR	11	32 569	33.8	0.86	0.43, 1.73	
HPD	2	8891	22.5	0.45	0.11, 1.87	

*per 100 000 person-days.
PD: pancreatoduodectomy, EHBD: extrahepatic bile duct resection, HR + EHBD: hepatic resection with extrahepatic bile duct resection, HR: hepatic resection, HPD: combined hepatectomy and pancreatoduodectomy. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

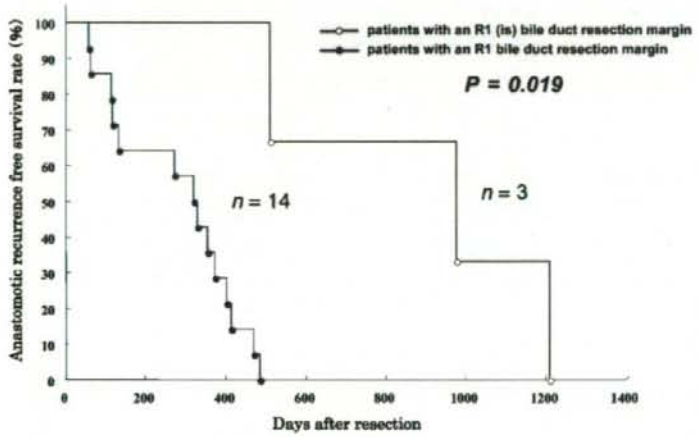

Fig. 3. Anastomotic recurrence-free survival rate of patients with a positive bile duct resection margin. Kaplan-Meier analysis revealed that the period until anastomotic recurrence at the bile duct resection margin after surgical resection in patients with a subepithelial invasive component bile duct resection margin (solid circles) was significantly shorter than that in patients with an intraductal carcinoma component bile duct resection margin (clear circles) ($P = 0.019$).

Table 7. Adujusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by bile duct resection margin status

	Adjusted HR†	95% CI
Total (214 cases)		
Bile duct resection margin status		
Negative	1.00	
R1 (is)	1.06	0.53, 2.10
R1	1.95	1.27, 3.00

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, and venous involvement. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

Correlation between bile duct resection margin status and anastomotic recurrence at the bile duct resection margin. In order to understand the background factors responsible for the difference in prognostic impact between R1 (is) and R1 bile duct resection margins, the correlation between bile duct resection margin status [R1 (is) *vs* R1] and anastomotic recurrence at the bile duct resection margin was examined by multivariate analysis. The risk of anastomotic recurrence in patients with an R1 bile duct resection margin was 4.5 times higher than that in patients with an R1 (is) bile duct resection margin (Table 8). In addition, the Kaplan-Meier method revealed that the period until anastomotic recurrence after surgical resection in patients with an R1 bile duct resection margin was significantly shorter than that in patients with an R1 (is) bile duct resection margin (Fig. 3, $P = 0.019$).

Discussion

Unlike several previously published studies analyzing the prognostic parameters in small series of patients with biliary tract carcinoma,[21–28] the present study examined in detail the clinicopathological parameters of 214 patients who underwent radical surgery with curative intent and had been strictly followed up at a single institution.

It has recently been reported that biliary tract carcinoma has a marked tendency for superficial extension by wide intraductal carcinoma[11–14] In this study, we defined the intraductal carcinoma component as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component. Surprisingly, an intraductal carcinoma component outside the main tumor nodule was significantly correlated with lower aggressiveness in the main tumor nodule:

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Table 8. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of anastomotic recurrence by bile duct resection margin status in patients with a positive bile duct resection margin

	No. of recurrent cases	Person-days	Adjusted HR†	95% CI
Bile duct resection margin status				
R1 (is)	3	22 029	1.00	
R1	14	25 237	4.48	1.09, 18.5

†Adjusted for operation day and type of surgical resection. R1 (is): resection margin with intraductal carcinoma component, R1: resection margin with subepithelial invasive component.

patients with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule (Table 1). Furthermore, patients with an intraductal carcinoma component were infrequently at the advanced stage when diagnosed (Table 1). During the preparation of this paper, Nakanishi *et al.*[29] reported that cases of extrahepatic bile duct carcinoma with intraepithelial spread showed a more differentiated histological grade, less deep invasion, infrequent portal vein or hepatic invasion and a better prognosis than cases without such spread. In addition, we demonstrated statistically significant inverse correlations between the intraductal carcinoma component on the one hand and lymphatic and perineural involvement and TNM stage on the other, whereas Nakanishi *et al.* failed to show such correlations. Moreover, we demonstrated an inverse correlation between the intraductal carcinoma component and tumor aggressiveness by both univariate and multivariate analyses in a larger cohort than that reported by Nakanishi *et al.*[29], whereas that report performed only univariate analysis.

We have revealed that human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models show strong adhesiveness to extracellular matrix proteins, which are components of the basement membrane of epithelial tissues, *in vitro*.[30] The expression patterns of cell-matrix adhesion molecules, such as integrins, in human cancer cell lines showing wide intraepithelial spreading in mouse inoculation models differ from those in human cancer cell lines that do not show such spreading.[30] In addition, it has been confirmed that there is a similar difference in the expression pattern of cell-matrix adhesion molecules between cancer cells showing, and not showing, such spreading in surgically resected clinical samples.[30] Cell-matrix adhesion molecules generally participate in cancer-stromal interactions and determine the invasiveness of human cancers.[31] Therefore, it is feasible that cancer cells showing wide intraepithelial spreading also show strong adhesiveness to the basement membrane of cancer nests and a less invasive tendency. This may be the reason why an intraductal carcinoma component was inversely correlated with aggressiveness in the main tumor nodule in this study. Although the molecular mechanism responsible for such an inverse correlation in biliary tract carcinoma needs to be further clarified, the presence of an intraductal carcinoma component may become an indicator of lower tumor aggressiveness. In fact, patients with an intraductal carcinoma component showed a significantly better prognosis than patients without such a component, irrespective of whether all patients from the present cohort or only patients who underwent complete resection were examined. The correlation between an intraductal carcinoma component and a favorable prognosis is consistent with the similar correlation observed in ductal carcinoma of the pancreas: the presence of an intraductal carcinoma component is reportedly a significantly good prognostic parameter for patients with invasive ductal carcinoma of the pancreas after surgical resection.[32–34]

On the other hand, the presence of an intraductal carcinoma component was significantly correlated with a positive bile duct resection margin (Table 4). Recently, Wakai *et al.* have reported that invasive carcinoma at the ductal resection margin appears to have a strong impact on patient survival, whereas residual carcinoma *in situ* does not, after surgical resection for extrahepatic bile duct carcinoma,[14] although they did not mention any background factors for the difference in prognostic impact between invasive carcinoma and carcinoma *in situ* at the ductal resection margin. We also defined two types of positive bile duct resection margin: R1 (is) (positive with only an intraductal carcinoma component) and R1 (positive with a subepithelial invasive component). An R1 bile duct resection margin was more frequently associated with poorer prognosis than a negative bile duct resection margin, whereas an R1 (is) bile duct resection margin was not more frequently associated with poorer prognosis than a negative bile duct resection margin (Tables 6 and 7). As in other malignancies, tumor recurrence after radical surgery for biliary tract carcinoma leads to eventual death.[26] Although Wakai *et al.* did not perform statistical analysis about anastomotic recurrence, the risk of anastomotic recurrence at the bile duct resection margin was significantly higher (Table 8), and the period until such recurrence was significantly shorter (Fig. 3), in patients with an R1 bile duct resection margin than in patients with an R1 (is) bile duct resection margin. Therefore, at least part of the difference in prognostic impact between R1 (is) and R1 bile duct resection margins is attributable to the difference in the risk of anastomotic recurrence.

However, the incidence of anastomotic recurrence in patients whose bile duct resection margin was positive [R1 (is) and R1] was only 26% (17 of 66 cases); some patients may die because of 'local recurrence' or distant metastasis before anastomotic recurrence becomes clinically obvious. Sakamoto *et al.* have proposed that anastomotic recurrence should be distinguished from 'local recurrence' derived from perineural invasion around the hepatic artery and/or involved regional nodes.[35] It is self-evident that all patients with an R1 (is) bile duct resection margin possess an intraductal carcinoma component that is inversely correlated with tumor aggressiveness, including perineural and lymphatic involvement and clinical stage. The intraductal carcinoma component may be partly responsible for the difference in prognostic impact between R1 (is) and R1 with reference not only to 'anastomotic recurrence' but also to 'local recurrence' derived from perineural invasion and/or involved regional nodes.

We analyzed both the intraductal carcinoma component and resection margin status in the same cohort and examined in detail the inconsistency of less tumor aggressiveness and a positive surgical margin in patients with intraductal carcinoma components. Surgeons are frequently required to decide the resection area based on the results of intraoperative histological diagnosis of bile duct resection margin status using frozen sections, and generally intend to achieve a negative bile duct resection margin. If the frozen section diagnosis is R1, the risk of death will be actually reduced if surgeons make efforts to achieve a negative bile duct resection margin. However, if the frozen section diagnosis is R1 (is), an intraductal carcinoma component is present and the prognosis for such patients will be

favorable. Moreover, if surgeons perform additional resection to achieve a negative bile duct resection margin, then the prognosis for patients whose initial bile duct resection margin is R1 (is) may not be improved significantly. Therefore, surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications.

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help to decrease the high percentage of missing values. Second, studies should ensure that standard methods and antibodies to assess CA 19-9 concentration are used and should report survival according to different baseline CA 19-9 concentrations. These measures will allow appropriate comparisons of different studies and practical conclusions to be drawn to enable appropriate patient stratification in future phase III trials.

*Gianpaolo Balzano, *Valerio Di Carlo*
Pancreas Unit, Department of Surgery, San Raffaele Scientific Institute, Milan, Italy
dicarlo.valerio@hsr.it

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Overexpression of HDACs: a prognostic marker for gastric cancer identified by tissue microarray

See Articles page 139

Acetylation and deacetylation of histones have important roles in transcriptional regulation in eukaryotic cells.[1] The acetylation status of histones is decided by the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs acetylate the ε-amino group of lysine residues on histones, thereby neutralising their positive charge and diminishing their ability to bind negatively charged DNA. An open chromatin configuration provides accessibility for transcription factors. Cross-talk between histone acetylation or methylation and DNA methylation has profound implications for gene expression. HDACs remove the acetyl groups, thereby allowing compacted chromatin to reform. So far, 18 HDAC isoforms have been identified and classified based on homology with yeast HDACs. HDACs also have many non-histone protein substrates, such as transcription factors and signal transduction mediators.

Histone acetylation is, along with DNA methylation, one of the most consistent epigenetic mechanisms of multistage human carcinogenesis.[2] Histone hypoacetylation affects the expression of genes involved in uncontrolled cell growth, differentiation, and apoptosis. The p21 gene is one of the best-studied targets of histone hypoacetylation in human cancers. In gastric cancer, overexpression at the mRNA and protein levels of HDAC isoform 1[3] has been reported in 25 samples of gastric-cancer tissue and in 71 samples of gastric-cancer tissue for HDAC isoform 2.[4] However, to my knowledge, there have been no published systematic reports about the expression levels of HDACs in large series of patients with gastric cancer.

In this issue of *The Lancet Oncology*, Weichert and colleagues[5] report an immunohistochemical study of the expression levels of HDAC isoforms 1, 2, and 3 in two cohorts of patients with gastric cancer (143 patients in a training group and 150 patients in a validation group). Initially, nuclear staining of HDACs in cancer cells was scored according to staining intensity and percentage of immunoreactive cells. After several grouping algorithms were tested, the simplest cut-off values were chosen for reproducibility, since clinicopathological and survival correlations were fairly robust. This system showed that concurrent overexpression of HDACs and overexpression of HDAC2 were significantly correlated with lymph-node metastasis and shorter survival. Immunohistochemical assessment of HDACs might therefore be of substantial prognostic value for gastric cancer, although the present system should be validated in appropriate prospective studies.

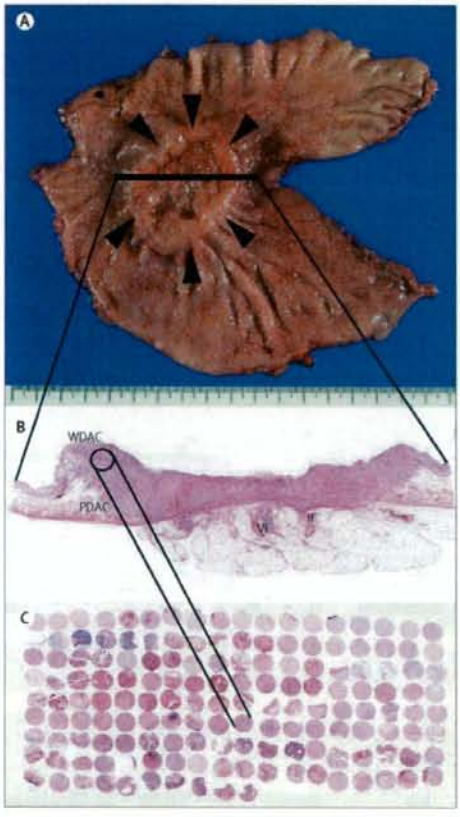

Figure: Tissue microarray block including a cylindrical core from a gastric cancer

(A) Macroscopic view of an advanced gastric cancer (arrowheads) in a distal gastrectomy specimen. (B) Histological heterogeneity is evident in a routine histological slide. WDAC=well-differentiated adenocarcinoma component. PDAC=poorly differentiated adenocarcinoma component. IF=invading front. VI=venous involvement. Haematoxylin-eosin staining. (C) A cylindrical core represents only some of the characteristics of this tumour. Haematoxylin-eosin staining. Construction of tissue microarray was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. Photos courtesy of Yukihiro Nakanishi, Pathology Division, National Cancer Center Research Institute, Tokyo, Japan.

HDAC inhibitors induce the expression of p21 and other cell-cycle regulators, pro-apoptotic proteins such as Bax and Bad, and repress the expression of metastasis-promoting proteins, such as matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), and pro-angiogenic factors such as vascular endothelial growth factor.[6] Healthy cells are relatively more resistant to HDAC-inhibitor-induced cell death. Therefore, HDAC inhibitors might be promising new anticancer drugs, and many are currently being tested in phase I or II clinical trials.[7] After appropriate validation, the present data obtained by Weichert and co-workers might help to predict therapeutic responses to HDAC inhibitors in patients with gastric cancer.

The immunohistochemical assessment system was established by Weichert's group with the use of tissue microarray. This technique allows rapid visualisation of molecular targets in thousands of tissue specimens at a time, and at the DNA, RNA, or protein level. Unlike methods that use conventional time-consuming molecular pathology, high-throughput molecular profiling of cancers by use of tissue microarray might be advantageous for screening diagnostic and treatment targets.[8,9] However, each cylindrical core constitutes only a very small portion of a tumour (figure), and thus carries only a small amount of molecular information. Weichert and colleagues took multiple cores from each tumour, and the cut-off values obtained from tissue microarray were validated in a large set of routine histological slides. This type of careful approach is strongly recommended when screening molecular targets using tissue microarray.

However, in spite of such careful validation, the success of screening with the use of tissue microarray might be restricted if immunoreactivity varies substantially in a tumour. Cancers frequently show histological heterogeneity—eg, well-differentiated and poorly differentiated components might be present simultaneously in a single tumour. During microscopic assessment of routine histological slides, some candidate target molecules show differences in immunohistochemical staining intensity or subcellular localisation between the well-differentiated and poorly differentiated components, or between the intraepithelial region and the invading front, even in a single tumour.[10,11] Such heterogeneity in staining patterns, which can be easily overlooked in tissue microarray studies, evokes speculation about the function of the candidate molecule and its participation in multistage carcinogenesis. Not only the efficiency of tissue microarray, but the abundant information obtained from routine histological slides will be of utmost importance in cancer research.

Yae Kanai
Pathology Division, National Cancer Center Research Institute, Tokyo, Japan
ykanai@ncc.go.jp

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Review Article

Alterations of DNA methylation and clinicopathological diversity of human cancers

Yae Kanai

Pathology Division, National Cancer Center Research Institute, Tokyo, Japan

Alterations of DNA methylation can account for the histological heterogeneity, reflected in the stepwise progression and complex biological characteristics of human cancers, that genetic alterations alone cannot explain. Analysis of DNA methylation status in tissue samples can be an aid to understanding the molecular mechanisms of multistage carcinogenesis. Human cancer cells show a drastic change in DNA methylation status, that is, overall DNA hypomethylation and regional DNA hypermethylation, which results in chromosomal instability and silencing of tumor-suppressor genes. Overexpression of DNA methyltransferase (DNMT) 1 is not a secondary result of increased cell proliferative activity but may underline the CpG island methylator phenotype of cancers. Splicing alteration of DNMT3B may result in chromosomal instability through DNA hypomethylation of pericentromeric satellite regions. Alterations of DNA methylation are observed even in the precancerous stage frequently associated with chronic inflammation and/or persistent viral infection or with cigarette smoking. Precancerous conditions showing alterations of DNA methylation may generate more malignant cancers. Aberrant DNA methylation is significantly associated with aggressiveness of cancers and poorer outcome of cancer patients. Genome-wide analysis of DNA methylation status based on array-based technology may identify DNA methylation profiles that can be used as appropriate indicators for carcinogenetic risk estimation and prognostication.

Key words: chromosomal instability, chronic inflammation, DNA methylation, DNMT1, DNMT3B, hepatocellular carcinoma, multistage carcinogenesis, precancerous condition, renal cell carcinoma, urothelial carcinoma

Microscopy of human cancers, which are considered to be genetically clonal lesions, frequently indicates histological heterogeneity (e.g. well, moderately or poorly differentiated carcinoma components are simultaneously observed even in tissue sections from a single patient). Such histological heterogeneity reflects the stepwise progression and complex biological characteristics of each tumor. Genetic alterations causing activation of oncogenes and inactivation of tumor suppressor genes cannot solely explain such histological heterogeneity of human cancers. Epigenetics has been defined as 'heritable changes in gene expression that are not due to any alteration in the DNA sequence'[1] and normally accounts for the diversity of phenotypes within cloned animals, monozygotic twins and single populations that genetics alone cannot explain.[2] Analysis of epigenetic alterations in tissue samples, in connection with the histological features of each cancer, may aid understanding of the molecular background of clinocopathological diversity in human cancers. DNA methylation is one of the most consistent and best-known epigenetic events in human cancers.

DNA methylation, a covalent chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides (Fig. 1a), plays important roles in chromatin organization and gene expression.[3] DNA methylation can directly impede the binding of transcription factors to their target sites, thus prohibiting the transcription of specific genes. Moreover, DNA methylation normally promotes a highly condensed heterochromatin structure, where active transcription does not occur, through recruitment of DNA-organizing proteins (Fig. 1b). DNA methylation is a stable modification that is inherited throughout cell divisions (Fig. 1c). When found within the promoter regions, DNA methylation prevents the reactivation of silent genes. This allows the daughter cells to retain the same expression pattern as the parent cells and is important for inactivation of the X chromosome and imprinting. Transposons and other parasitic elements have been acquired in the mammalian genome over time, and make up the repetitive sequences in the intergenic and intragenic regions of DNA. The activation of these parasitic elements can allow for the movement of these elements within the

Correspondence: Yae Kanai, MD, PhD, Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Email: ykanai@ncc.go.jp

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genome. To preserve the integrity of the genome, DNA methylation persistently silences such parasitic elements.[4]

Murine *DNA methyltransferase 1 (Dnmt 1)* cDNA was cloned in 1988 and its C-terminal domain was found to show striking similarities to the catalytic methyltransferase domain of bacterial type II DNA cytosine methyltransferases.[5] Homologs of DNMT1 have been found in nearly all eukaryotes that have DNA bearing 5-methylcytosine, but not in those that lack it. Until the identification of DNA methyltransferase (DNMT) 2,[6] DNMT3A and DNMT3B[7] in 1998, DNMT1 (EC2.1.1.37) had been the only known DNMT, and is currently the major and best-known of this enzyme family. Embryos of *Dnmt1 –/–* mice, which have genome-wide DNA hypomethylation, are stunted, show delayed development, and do not survive past mid-gestation,[8] indicating that DNA methylation is essential for the development of mammals.

The C-terminal catalytic domain of DNMT transfers methyl groups from S-adenosyl-L-methionine (AdoMet) to cytosines (Fig. 1a).[9] Critical dietary components leading to synthesis of AdoMet include folate, vitamins B_6 and B_{12}, methionine and choline. The C-terminal catalytic domain of DNMT is characterized by the presence of five conserved amino acid motifs, namely I, IV, VI, IX and X (Fig. 1d).[9] Motifs I and X are filed together to form most of the binding site for AdoMet. Motif IV contains the prolylcysteinyl dipeptide that provides the thiolate at the active site. Motif VI contains the glutamyl residue that protonates the 3 position of the target cytosine. Motif IX has a role in maintaining the structure of the target recognition domain.

The N-terminal regulatory domain of DNMT1 contains a proliferating cell nuclear antigen (PCNA)-binding domain, a nuclear localization signal, a cysteine-rich alpha thalassemia and retardation on the X (ATRX) zinc finger DNA-binding motif, and a polybromo homology domain targeting DNMT1 to the replication foci (Fig. 1d).[10] Thus DNMT1 forms the core of the DNA replication machinery complex. In addition to methyltransferase activity, interaction with DNMT1-associated protein (DMAP) 1,[11] E2F1,[12] histone deacetylase (HDAC) 1 and 2 and methyl CpG binding proteins (MBD)[13] through the N-terminal regulatory domain makes DNMT1 a crucial element of the transcription suppression complex.

The preference of DNMT1 for hemi-methylated over unmethylated substrates *in vitro*[14] and its targeting of replication foci[15] are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand. Thus, DNMT1 has been recognized as the 'maintenance' DNMT (Fig. 1c). Although DNMT2 contains the full set of conserved motifs of the C-terminal catalytic domain, it lacks the N-terminal regulatory domain characteristic of eukaryotic DNMT (Fig. 1d). The methyltransferase activity of the recombinant DNMT2 protein is weak *in vitro* and *in vivo*. DNMT3A and DNMT3B also contain the full set of conserved motifs of the C-terminal catalytic domain, and their N-terminal regulatory domains are divergent on the N-terminal side of the cysteine-rich ATRX zinc finger DNA-binding motif (Fig. 1d). DNMT3A and DNMT3B show *de novo* DNA methylation activity (Fig. 1c) *in vitro*.[16] Pericentromeric satellite regions are considered to be one of the specific targets of DNMT3B, because Dnmt3B–/– mice lack DNA methylation in such regions and die *in utero*.[16] Germline mutations of the *DNMT3B* gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation on pericentromeric satellite regions.[17] Because *de novo* methylation of CpG islands has actually been observed in human fibroblasts overexpressing DNMT1,[18] DNMT1 is capable of *de novo* DNA methylation activity *in vivo* as well as having a maintenance function, and DNA methylation status may be determined on the basis of cooperation between DNMT1 and the DNMT3 family *in vivo*.[19] DNMT3L lacks conserved motifs of the catalytic domain but is otherwise closely related to the N-terminal regulatory domain of DNMT3A and DNMT3B (Fig. 1d) and cooperates with the DNMT3 family to establish an imprinting pattern.[20]

MBD are one of mediators of cross-talk between DNA methylation and another major epigenetic event, histone modification. Until 1998, MeCP2 had been the only functionally defined MBD. When MeCP2 binds to methylated CpG dinucleotide, its transcriptional repression domain recruits a co-repressor complex containing Sin 3A and HDAC, resulting in compaction of the chromatin and stable repression of the target gene.[21,22] Later, MBD1, MBD2, MBD3 and MBD4 were identified. MBD1 interacts with histone H3 methyltransferase SETDB1.[23] MBD2[24] and MBD3[25] are involved in another HDAC complex, Mi-2/NuRD. MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites.[26]

DNA METHYLATION AND HUMAN CANCERS

In comparison with normal cells, human cancer cells show a drastic change in DNA methylation status, generally exhibiting global DNA hypomethylation and accompanying region-specific hypermethylation.[27–30] Because 5-methylcytosine is deaminated to thymine, DNA hypermethylation facilitates gene mutation in human cancers. DNA hypomethylation in cancer cells causes chromatin decondensation and chromosomal rearrangements that may result in chromosomal instability. Moreover, DNA hypermethylation of CpG islands near the promoter regions silences specific genes including tumor suppressor genes in cooperation with histone modification:[31] hypermethylation of CpG islands in the promoter regions of tumor-suppressor genes in cancer cells is associated with

deacetylation of histones H3 and H4, loss of histone H3, lysine 4 (H3K4) methylation, and gain of H3K9 methylation (Fig. 1b).

A reduction of DNMT1 activity in ApcMin mice due to heterozygosity of the *Dnmt1* gene, in conjunction with treatment using the DNMT inhibitor 5-aza-deoxycytidine, reduces the average number of intestinal adenomas.[32] In contrast, genomic hypomethylation in *Nf1+/– p53+/–* (NPcis) mice due to the introduction of a hypomorphic allele of *Dnmt 1* (Dnmt1Chip/–) induces sarcomas at an earlier age in comparison with NPcis littermates possessing normal levels of DNA methylation (Dnmt1Chip/+).[33] The loss of heterozygosity (LOH) rate is increased in hypomethylated cells in Dnmt1Chip/– mice. Chromosomal instability accompanied by activation of endogenous retroviral elements has also been observed in Dnmt1Chip/– mice.[34] These observations in genetically engineered animals clearly demonstrate a causal relationship between alterations of DNA methylation and human cancers. Correlation between the etiological backgrounds of human cancers and alterations of DNA methylation, however, can be clarified only by analysis of clinical samples.

In order to determine the significance of DNA methylation alterations during multistage carcinogenesis, DNA methylation status should be analyzed in a range of tissue samples from precancerous conditions to malignant states (Fig. 2). Such empirical data are indispensable for clinical application of DNA methylation to carcinogenetic risk estimation, early diagnosis, prognostication, prevention and therapy. Therefore the following sections describe the results obtained by analysis of DNA methylation status in tissue samples for which the clinicopathological characteristics have been strictly determined.

HEPATOCARCINOGENESIS IN LIVERS DAMAGED BY HEPATITIS VIRUS INFECTION

Alterations of DNA methylation in precancerous conditions

The majority of hepatocellular carcinomas (HCC) are associated with HBV or HCV infection. Clonal expansion of hepatocytes is initiated during the regeneration process in damaged livers; a clonal integration pattern of HBV is evident in each cirrhotic nodule. Therefore, chronic hepatitis and liver cirrhosis are considered to be precancerous conditions. Small nodular lesions of early-stage HCC first develop in livers with chronic hepatitis and cirrhosis, and then progressed HCC often emerge within early-stage HCC nodules (nodule-in-nodule-type HCC). Thus, macro- and microscopically, HCC represent a typical scenario of multistage carcinogenesis.[35]

The LOH on chromosome 16 has been frequently detected on classic restriction fragment length polymorphism using Southern blot in HCC that are poorly differentiated, large in size, and associated with metastasis.[37] Therefore, this seems to be a late event during multistage hepatocarcinogenesis. At the time of these discoveries, only a few molecular events in the earlier stage of hepatocarcinogenesis were known. But studies using classic Southern blot with a DNA methylation-sensitive restriction enzyme frequently showed alterations of DNA methylation at multiple loci on chromosome 16 even in non-cancerous liver tissues with chronic hepatitis or cirrhosis, unlike normal liver tissues obtained from patients with liver metastases from primary colon cancer.[38] This was one of the earliest reports of alterations of DNA methylation in the precancerous stage. Because the molecular weight of DNA fragments digested using a DNA methylation-sensitive restriction enzyme in HCC was higher than that in precancerous conditions, and the intensity of larger-sized bands was increased in HCC in comparison with precancerous conditions, the numbers of methylated CpG dinucleotides and cells having DNA hypermethylation may increase progressively as precancerous conditions develop into HCC.[38] The incidence of DNA hypermethylation on chromosome 16 was significantly correlated with higher histological grade, portal vein involvement and intrahepatic metastasis of HCC.[38] The presence of DNA hypermethylation in both precancerous conditions and progressed HCC suggests that aberrant DNA methylation is one of the earliest molecular events during hepatocarcinogenesis and also participates in malignant progression.

Silencing of tumor suppressor genes

The *E-cadherin* gene is located on 16q22.1 near the aforementioned hot spots of both DNA hypermethylation and LOH in HCC. E-cadherin acts as a Ca^{2+}-dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells.[39] Cell–cell adhesion determines cell polarity and participates in histogenesis. The mutual adhesiveness of cancer cells is significantly weaker than that of normal cells, and this allows cancer cells to disobey the social order, resulting in destruction of histological architecture, which is a morphological hallmark of malignant tumors. The *E-cadherin* gene is a tumor suppressor gene that can be silenced by a two-hit mechanism consisting of LOH and gene mutation in cancers such as signet-ring cell carcinoma of the stomach[40] and lobular carcinoma of the breast,[41] in which cancer cells completely lose their mutual adhesiveness even in the *in situ* carcinoma stage. In contrast, reduced expression of E-cadherin is believed to trigger the release of cancer cells from primary cancer nests, resulting in cancer invasion and metastasis.[42] Significant correlations between reduced expression of E-cadherin and poor prognosis have been

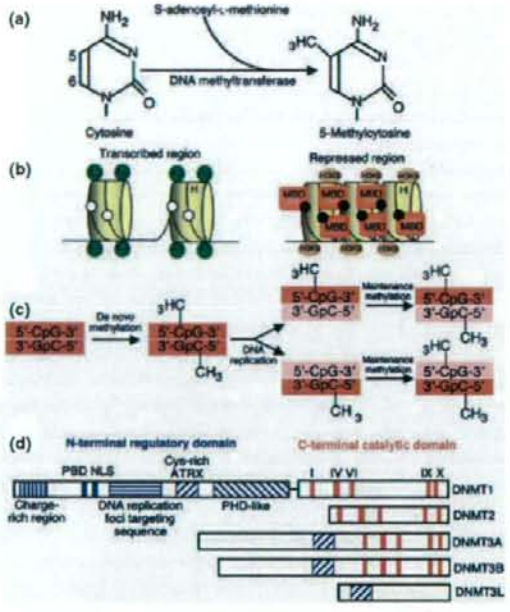

Figure 1 DNA methylation in mammals. (**a**) DNA methylation is a covalent chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides. DNA methyltransferases (DNMT) transfer methyl groups from S-adenosyl-L-methionine to cytosines. (**b**) DNA methylation normally promotes a highly condensed heterochromatin structure, in which active transcription does not occur, through recruitment of DNA-organizing proteins including histone deacetylase complex and methyl CpG binding proteins (MBD). The repressed regions are associated with deacetylation of histones H3 and H4 and gain of histone H3, lysine 9 (H3K9) methylation. H, histone octamer; (○) unmethylated CpG dinucleotides; (●) methylated CpG dinucleotides. (**c**) DNA methylation is a stable modification that is inherited throughout cell divisions (maintenance methylation). The preference of maintenance DNMT for hemi-methylated over unmethylated substrates and its targeting of replication foci are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand. *De novo* methylation occurs by *de novo* DNMT during the development of mammals and carcinogenesis. (**d**) Structure of DNMT. The C-terminal catalytic domain is characterized by the presence of conserved motifs I, IV, VI, IX and X. The N-terminal regulatory domain of DNMT1 contains a proliferating cell nuclear antigen-binding domain (PBD), a nuclear localization signal (NLS), a cysteine-rich alpha thalassemia and retardation on the X (ATRX) zinc finger DNA-binding motif, and a polybromo homology domain (PHD) targeting DNMT1 to the replication foci.

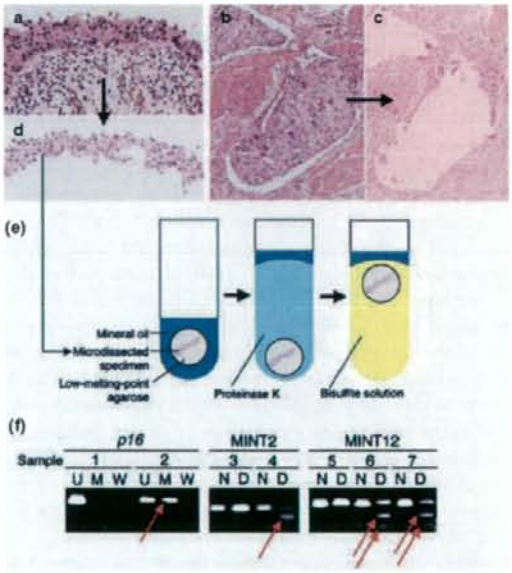

Figure 2 Analysis of DNA methylation status in tissue specimens. Tissue sample of carcinoma *in situ* (**a**) before and (**d**) during microdissection, and that of invasive carcinoma (**b**) before and (**c**) after microdissection. (**e**) Microdissected specimens were subjected to agarose bead-embedded methylation-specific polymerase chain reaction (MSP),[35] which was originally developed for analysis of DNA methylation in tiny tissue samples. (**f**) Examples of the results of MSP for the *p16* gene and combined bisulfite restriction enzyme analysis (COBRA) for MINT 2 and 12 clones. Polymerase chain reaction products yielded by primer sets for methylated (M), unmethylated (U) and unmodified wild-type (W) DNA and digested (D) and non-digested (N) DNA fragments using methylation-sensitive restriction enzymes are shown. Arrows, methylated DNA fragments.

reported in patients with cancers.[42] The promoter region of the *E-cadherin* gene contained DNA methylation in human cancer cell lines lacking E-cadherin expression, and E-cadherin expression was induced after treatment with the DNMT inhibitor 5-azacytidine in such cell lines.[43] Thus, following the *retinoblastoma* (*RB*) and *von Hippel-Lindau* (*VHL*) genes, the *E-cadherin* gene became the third example of a tumor suppressor gene that is silenced by DNA hypermethylation.[43] When assessed on Southern blot, DNA hypermethylation around the promoter region of the *E-cadherin* gene can be frequently detected even in non-cancerous liver tissues showing chronic hepatitis or cirrhosis.[44] Heterogeneous E-cadherin expression in non-cancerous liver tissues showing chronic hepatitis or cirrhosis, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation.[44] A significant correlation between DNA hypermethylation around the promoter region and reduced expression of E-cadherin was found in HCC.[44] This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in a cohort of clinical tissue samples. DNA hypermethylation around the promoter region may participate in hepatocarcinogenesis through reduction of E-cadherin expression,

resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

The *hypermethylated in cancer (HIC)-1* gene at the D17S5 locus (17q13.3) was the first tumor suppressor gene to be identified in commonly methylated chromosomal loci in human cancers;[45] mice with germ line disruption of one allele of *Hic1* developed spontaneous malignant tumors.[46] DNA hypermethylation at the D17S5 locus was frequently detectable in non-cancerous liver tissues showing chronic hepatitis or cirrhosis,[47] as well as in stomach mucosa showing intestinal metaplasia.[48] Southern blot using a DNA methylation-sensitive restriction enzyme suggested that the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation might increase progressively as precancerous conditions develop into HCC.[47] The expression level of HIC-1 mRNA in non-cancerous liver that had chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissues, and was further decreased in HCC.[47]

Alterations of DNA methylation and chromosomal instability

The hot spot for DNA hypermethylation in HCC corresponds to a previously reported hot spot of LOH on chromosome 16.[38] It remains to be clarified whether alterations of DNA methylation might predispose the locus to allelic loss, or whether common or different causes facilitate both alterations of DNA methylation and LOH at certain loci. But classic Southern blot clearly showed that DNA hypermethylation precedes LOH at the same chromosomal loci during hepatocarcinogenesis: DNA hypermethylation was detected in bulk non-cancerous liver tissues showing chronic hepatitis or cirrhosis, in which LOH has never been detected using the same method.

Recently, microdissection techniques and polymerase chain reaction (PCR) using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissues. LOH has been reported even in microdissected specimens from dysplastic lesions adjacent to cancers. In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, in microdissected specimens obtained from pseudo-lobules and regenerative nodules in non-cancerous liver tissues having chronic hepatitis or cirrhosis and HCC, LOH and microsatellite instability were examined using multiple microsatellite markers, and the DNA methylation status of multiple C-type CpG islands[49] that are known to be methylated in a cancer-specific, but not age-dependent manner, was examined on methylation-specific PCR and combined bisulfite restriction enzyme analysis.[50,51]

LOH was never detected in normal liver tissues obtained from patients with liver metastases from primary colon cancers or in non-cancerous liver tissues having no remarkable histology from patients with HCC (Fig. 3a).[51] The incidence of LOH in chronic hepatitis or liver cirrhosis stages was almost the same (Fig. 3a). Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissues obtained from patients with liver metastases from primary colon cancers, DNA hypermethylation was found even in non-cancerous liver tissues having no remarkable histological features obtained from patients with HCC, in which LOH was never detected (Fig. 3a).[51] This phenomenon might be at least partly attributable to hepatitis viral infection. HBV-DNA is integrated into the cellular genome, and the integrated viral DNA is known to alter the DNA methylation status in several adjacent cellular genes and DNA segments.[52] The incidence of DNA hypermethylation on CpG islands overwhelmed that of LOH at all stages of chronic hepatitis, liver cirrhosis and HCC. Thus aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using PCR-LOH and microdissection. The low incidence of microsatellite instability in Japanese patients with HCC[53] (Fig. 3a) was compatible with absence of silencing of the *human MutL homologue 1* (*hMLH1*) gene by DNA hypermethylation during hepatocarcinogenesis.[51]

Overexpression of DNMT1

Abnormalities of DNMT underlying alterations of DNA methylation was examined during hepatocarcinogenesis. Mutational inactivation of the *DNMT1* gene that can potentially cause genome-wide alterations of DNA methylation was never detected in HCC or in stomach cancers, whereas colorectal cancers infrequently had mutations of the *DNMT1* gene, including a mutation resulting in deletion of the whole catalytic domain due to a premature stop codon.[54] Mutational inactivation of the *DNMT1* gene may be a rare event during human carcinogenesis. In contrast, the expression level of DNMT1 mRNA was significantly higher even in non-cancerous liver tissues having chronic hepatitis or cirrhosis than in normal liver tissues, and was even higher in HCC (Fig. 3b).[55,56] The incidence of DNMT1 protein overexpression in HCC is significantly correlated with poorer tumor differentiation and portal vein involvement (Fig. 3c).[57] Moreover, the recurrence-free and overall survival rates of patients with HCC that has overexpression of DNMT1 protein are significantly lower than those of patients with HCC that do not (Fig. 3d).[57] Immunohistochemistry of DNMT1 in liver biopsy specimens obtained for histological diagnostic purposes and/or hepatectomy specimens may become a useful tool for prognostication in individual clinical cases.

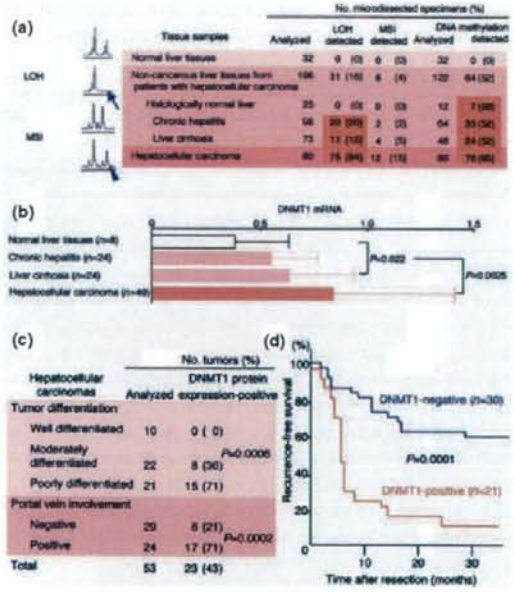

Figure 3 Alterations of DNA methylation during hepatocarcinogenesis. (**a**) Loss of heterozygosity (LOH; arrow) and microsatellite instability (MSI; arrowhead) were examined using 39 microsatellite markers; and DNA methylation status on 8 C-type CpG islands was examined on methylation-specific polymerase chain reaction and combined bisulfite restriction enzyme analysis of microdissected tissue specimens.[51] (**b**) The expression level of DNA methyltransferase 1 (DNMT1) mRNA was significantly higher even in non-cancerous liver tissues that had chronic hepatitis or cirrhosis than in normal liver tissues, and was even higher in hepatocellular carcinomas.[55,56] (**c**) DNMT1 protein expression in hepatocellular carcinomas was significantly correlated with poorer tumor differentiation and portal vein involvement.[57] (**d**) Recurrence-free survival rate of patients whose hepatocellular carcinomas had protein overexpression of DNMT1 was significantly lower than that of patients whose hepatocellular carcinomas did not.[57]

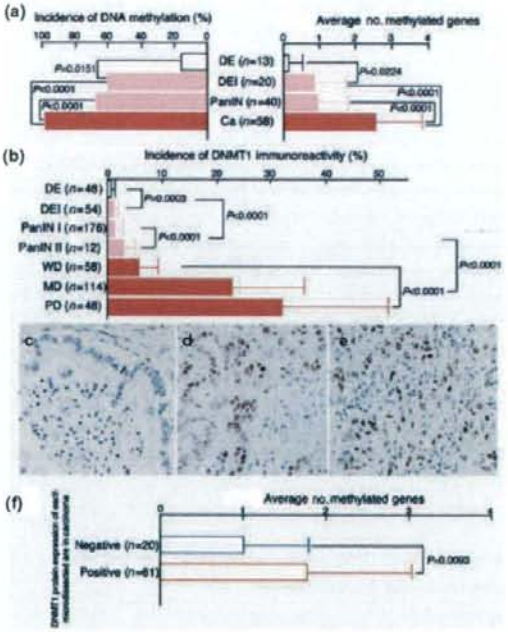

Figure 5 (**a**) Alterations of DNA methylation during pancreatic carcinogenesis. When DNA methylation status of the *p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, E-cadherin* and *DAPK-1* tumor-related genes was examined in microdissected specimens, the incidence of DNA hypermethylation of at least one of the genes and the average number of methylated genes were significantly higher in peripheral pancreatic duct epithelia with an inflammatory background (DEI) and pancreatic intra-epithelial neoplasia (PanIN) than in peripheral pancreatic duct epithelia without an inflammatory background (DE), and were further increased in ductal carcinomas (Ca).[70] (**b**) Incidence of nuclear DNA methyltransferase 1 (DNMT1) immunoreactivity was significantly elevated in DEI and PanIN than in DE.[71] The incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of PanIN dysplasia (PanIN I vs Pan IN II). The incidence of nuclear DNMT1 immunoreactivity was significantly higher in ductal carcinomas than in PanIN, and was associated with poorer differentiation of ductal carcinomas (MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; WD, well-differentiated adenocarcinoma). Heterogeneity of DNMT1 protein expression among components having different grades of histological differentiation was observed in a representative cancer from a single patient.[71] (**d**) Moderately and (**e**) poorly differentiated adenocarcinoma components had a higher incidence of DNMT1 immunoreactivity than (**c**) the well-differentiated adenocarcinoma component. (**f**) The average number of methylated tumor-related genes in microdissected specimens of ductal carcinomas was significantly correlated with the expression level of DNMT1 protein examined on immunohistochemistry in the precisely microdissected areas.[70]

Aberrant splicing of DNMT3B

Although DNA hypomethylation on pericentromeric satellite regions, such as satellites 2 and 3, was frequently detected in both non-cancerous liver tissues having chronic hepatitis or cirrhosis and HCC (Fig. 4a),[56] and such regions are one of the target sequences of DNMT3B, no mutation of any coding exon of the *DNMT3B* gene was detected in the examined HCC.[58] The total level of DNMT3B mRNA was higher in HCC than in the corresponding non-cancerous liver tissues (Fig. 4b).[56] Thus, it is unlikely that reduced expression of DNMT3B simply causes DNA hypomethylation in these regions during hepatocarcinogenesis. There are four splice variants in the C-terminal catalytic domain of DNMT3B. DNMT3B3 possesses the N-terminal region and conserved methyltransferase motifs I, IV, VI, IX and X (Fig. 4c) and its DNMT activity has been confirmed *in vitro*.[59] Data obtained on splice-variant-specific quantitative reverse transcription–PCR have indicated that the major variant in normal liver

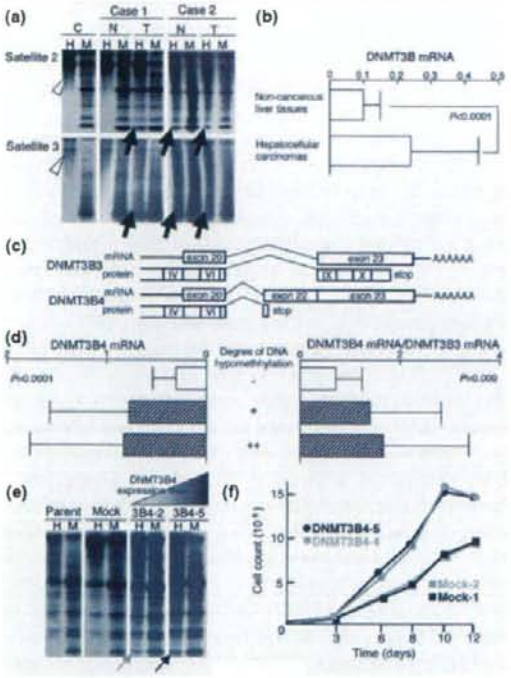

Figure 4 Overexpression of DNA methyltransferase 3B4 (DNMT3B4) associated with DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis. (**a**) Although satellites 2 and 3 were fully methylated in normal liver tissue obtained from a patient with liver metastasis from primary colon cancer (C, arrowheads), DNA hypomethylation on satellites 2 and 3 was frequently detected in both non-cancerous liver tissues that had chronic hepatitis or cirrhosis (N) and hepatocellular carcinomas (T).[56] H, methylation-sensitive restriction enzyme HpaII; M, methylation-non-sensitive restriction enzyme Msp I. Arrows, DNA hypomethylation. (**b**) The total level of DNMT3B mRNA was higher in hepatocellular carcinomas than in the corresponding non-cancerous liver tissue.[56] Thus, it is unlikely that reduced expression of DNMT3B causes DNA hypomethylation in these regions during hepatocarcinogenesis. (**c**) Structure of splice variants of DNMT3B, DNMT3B3 and DNMT3B4. (**d**) The level of DNMT3B4 mRNA and the ratio of DNMT3B4 mRNA to DNMT3B3 mRNA in non-cancerous liver tissues obtained from patients with hepatocellular carcinomas and in hepatocellular carcinomas were significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions.[58] +, smaller fragments detected in the *Hpa* II digest compared with the *Hpa* II digest of normal liver tissues; ++, *Hpa* II digest had the same hybridization pattern as the *Msp* I digest of its own and normal liver tissues. (**e**) DNA hypomethylation on satellite 2 was observed in transfection of human epithelial 293 cells with DNMT3B4 cDNA (clones 3B4-2 and 3B4-5) compared to mock transfectant (Mock) and parent 293 cells (Parent).[58] H; Hpa II digest; M, Msp I digest. (**f**) The growth rate of DNMT3B4 transfectants (clones DNMT3B4-4 and 3B4-5) was approximately double that of mock-transfectants (clones Mock-1 and Mock-2) soon after the introduction of DNMT3B4,[61] when chromosomal instability may not yet have accumulated.

tissues is DNMT3B3.[58] In contrast, DNMT3B4 probably does not show DNMT activity because it lacks the conserved methyltransferase motifs IX and X (Fig. 4c), although it retains the N-terminal domain required for targeting to pericentromeric satellite regions. Normal liver tissues showed only a trace level of DNMT3B4 expression.[58] The level of DNMT3B4 mRNA in non-cancerous liver tissues obtained from patients with HCC and in HCC was significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions (Fig. 4d).[58] In addition, the ratio of DNMT3B4 mRNA to DNMT3B3 mRNA in non-cancerous liver tissues obtained from patients with HCC and in HCC was also significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions (Fig. 4d).[58] DNMT3B4 lacking DNMT activity may compete with the major variant, DNMT3B3, for targeting to pericentromeric satellite regions. Then DNMT3B4 was introduced into human epithelial 293 cells, which express a significant level of DNMT3B3 mRNA and a trace level of endogenous DNMT3B4 mRNA. DNA demethylation on satellite 2 was observed in the DNMT3B4 transfectants, depending on the expression level of myc-tagged DNMT3B4 (Fig. 4e).[58] Satellite regions are abundant in pericentromeric heterochromatin DNA on chromosomes 1, 9 and 16. In fact, frequent chromosome 1q copy gain with a pericentromeric breakpoint has been reported in HCC having DNA hypomethylation on satellite 2.[60] DNMT3B4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis.

The growth rate of DNMT3B4 transfectants was approximately double that of mock-transfectants soon after the introduction of DNMT3B4 (Fig. 4f),[61] when chromosomal instability may not yet have accumulated. It was assumed that this change was caused by altered gene expression. Although the majority of the genes that were upregulated in DNMT3B4 transfectants were implicated in interferon signaling,[61] genes that encoded interferons themselves were not upregulated. Signal transducer and activator of transcription (STAT) 1,[61] which acts as an effector of interferon signaling, has been listed as one of the upregulated genes in DNMT3B4 transfectants. A significant correlation between the expression levels of DNMT3B4 and STAT1 mRNA was confirmed in tissue specimens of HCC.[61] Overexpression of DNMT3B4 is involved in multistage carcinogenesis not only by inducing chromosomal instability but also by affecting the expression of specific genes.

Altered expression of methyl CpG binding proteins

Although many researchers have focused on cross-talk between DNA methylation and histone modification,

abnormalities of MBD in human cancers do not seem to have attracted much attention. The expression level of MeCP2 mRNA in HCC with portal vein involvement is significantly lower than that in HCC without such involvement,[56] suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCC. Reduced MBD2 mRNA expression has been observed in HCC,[56] as well as in colorectal and stomach cancers,[62] suggesting that reduced MBD2 expression may be associated with a particular step in human carcinogenesis. The expression level of MBD4 mRNA in HCC is significantly lower than that in the corresponding non-cancerous liver tissues and is significantly correlated with poorer tumor differentiation and portal vein involvement.[56] Reduced MBD4 expression may result in frequent C-T transitions in tumor suppressor genes.

VIRUS INFECTION-ASSOCIATED CARCINOGENESIS IN THE STOMACH AND THE UTERINE CERVIX

In immunohistochemistry for DNMT1, nuclear immunoreactivity was not detected in any of the non-cancerous epithelia, except in proliferative zones, but was frequently found in stomach cancers.[63] DNMT1 overexpression, at both the mRNA[64] and protein[63] levels, correlated significantly with poorer tumor differentiation and with CpG island methylator phenotype (CIMP), defined by frequent DNA hypermethylation of C-type CpG islands,[65] in stomach cancers. The *hMLH1*, *thrombospondin-1* (*THBS-1*) and *E-cadherin* genes may be targets for overexpressed DNMT1 in stomach cancers.[63] Four percent of the examined patients with stomach cancers had EBV infection, a potential etiological factor in gastric carcinogenesis, in their cancer cells, and all cancers with EBV infection had DNMT1 protein overexpression.[63] Induction of latent membrane protein 1 of EBV has been reported to induce overexpression of DNMT1 in cultured cancer cells.[66] EBV infection in stomach cancers was associated with marked accumulation of DNA hypermethylation of C-type CpG islands.[63] With respect to stomach carcinogenesis, *Helicobacter pylori* infection, another etiological factor, is known to strongly promote regional DNA hypermethylation.[67]

Cervical intra-epithelial neoplasia (CIN) is a precursor lesion for squamous cell carcinoma of the uterine cervix closely associated with HPV infection. DNMT1 protein expression is increased even in low-grade CIN relative to normal squamous epithelium, and further increased in higher-grade CIN and squamous cell carcinomas of the uterine cervix.[68] HPV-16 E7 protein has been reported to associate directly with DNMT1 and stimulate the enzyme activity of DNMT1 *in vitro*,[69] and accumulation of DNA hypermethylation on tumor-related genes has also been observed during cervical carcinogenesis.

PANCREATIC CARCINOGENESIS ASSOCIATED WITH PERSISTENT INFLAMMATION

Cumulative DNA methylation of tumor-related genes

In the same way that HCC are preceded by chronic hepatitis, ductal carcinomas frequently emerge in pancreases damaged by chronic pancreatitis. Therefore, at least a proportion of peripheral pancreatic duct epithelia with an inflammatory background may be at the precancerous stage. DNA methylation status of the *p14*, *p15*, *p16*, *p73*, *adenomatous polyposis coli* (*APC*), *hMLH1*, *O-6-methylguanine-DNA methyltransferase* (*MGMT*), *breast cancer 1* (*BRCA1*), *glutathione S-transferase pi* (*GSTP1*), *tissue inhibitor of metalloproteinase 3* (*TIMP-3*), *E-cadherin*, and *death-associated protein kinase 1* (*DAPK-1*) tumor-related genes was examined on agarose bead-embedded methylation-specific PCR, which had been developed for DNA methylation analysis of tiny microdissected tissue specimens (Fig. 2e).[35] The incidence of DNA hypermethylation of at least one of the genes and the average number of methylated genes were significantly higher in peripheral pancreatic duct epithelia with an inflammatory background and in another precancerous lesion, pancreatic intraepithelial neoplasia (PanIN), than in peripheral pancreatic duct epithelia without an inflammatory background, and was further increased in ductal carcinomas (Fig. 5a).[70] The *BRCA1*, *APC*, *p16* and *TIMP-3* genes are frequently methylated in ductal carcinomas of the pancreas.[70]

Overexpression of DNMT1

When examined on immunohistochemistry, the incidence of nuclear DNMT1 immunoreactivity was significantly elevated in peripheral pancreatic ductal epithelia with an inflammatory background and PanIN than in peripheral pancreatic ductal epithelia without an inflammatory background (Fig. 5b).[71] With respect to inflammation-related carcinogenesis,[72] treatment with the cytokine interleukin-6 has been reported to induce overexpression of DNMT1 in cultured cells.[73] The incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of PanIN dysplasia (Fig. 5b), being significantly higher in invasive ductal carcinomas than in PanIN, and was associated with poorer differentiation of invasive ductal carcinomas (Fig. 5b–e).[71] Protein overexpression of DNMT1 in ductal carcinomas is significantly correlated with the extent of cancer invasion to surrounding organs and with advanced stage,[71] suggesting that overexpression of DNMT1 is associated with aggressiveness of pancreatic cancers. Moreover, patients with ductal carcinomas of the pancreas