Promoter hypermethylation of *DAP-kinase* is associated with poor survival in primary biliary tract carcinoma patients

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To clarify the clinicopathological significance of promoter hypermethylation of tumor suppressor and tumor-related genes in biliary tract carcinomas, we examined the promoter methylation status of multiple genes in primary biliary tract carcinomas. These consisted of carcinomas of the bile duct, gallbladder, and duodenal ampulla. Surgical specimens were obtained from a total of 37 patients with biliary tract carcinoma. The cohort consisted of 23 patients with bile duct carcinoma, 9 patients with gallbladder carcinoma, and 5 patients with ampullary carcinoma. The methylation status of *CHFR*, *DAP-kinase*, *E-cadherin*, *hMLH1*, *p16*, *RASSF1A*, and *RUNX3* was examined by methylation-specific polymerase chain reaction (MSP). The correlation between methylation status and clinicopathological characteristics was then assessed. The methylation frequencies of *CHFR*, *DAP-kinase*, *E-cadherin*, *hMLH1*, *p16*, *RASSF1A*, and *RUNX3* genes were 16.2%, 21.4%, 27.0%, 8.1%, 24.3%, 27.0%, and 56.8%, respectively, in primary biliary tract carcinomas. The number of methylated genes per sample was 2.17±0.28 (average±SD) in bile duct carcinomas, 1.80±0.97 in ampullary carcinomas, and 0.89±0.35 in gallbladder carcinomas, with a statistically significant difference between bile duct carcinomas and gallbladder carcinomas (*P*=0.02). As for clinicopathological significance, patients with a methylated *RUNX3* promoter were significantly older than those with unmethylated *RUNX3* (*P*=0.01), and *DAP-kinase* methylation was more frequent in poorly differentiated tumors than in well to moderately differentiated ones (*P*=0.04). The overall survival rate was significantly lower in patients with methylated *DAP-kinase* (*P*=0.009) or *RUNX3* (*P*=0.034) compared to those with unmethylated genes. Furthermore, *DAP-kinase* methylation-positive status was independently associated with poor survival in multivariate analyses (hazard ratio=8.71, *P*=0.024). A significant proportion of primary biliary tract carcinomas exhibited promoter hypermethylation of tumor suppressor and tumor-related genes, although bile duct carcinomas are more prone to being affected by promoter methylation than are gallbladder carcinomas. Hypermethylation of *DAP-kinase* appears to be a significant prognostic factor in primary biliary tract carcinomas. (Cancer Sci 2004; 95: 736–740)

Biliary tract carcinoma is a disease with a poor prognosis. The 5-year survival rate is less than 25% for intra- and extrahepatic bile duct carcinoma, and 32% to 61% for gallbladder carcinoma, even after radical resection of the tumor.[1–3] There is no effective therapy for biliary tract carcinomas except surgical resection. Moreover, the molecular-biological mechanisms of the development of biliary tract carcinomas are less well understood than those of carcinomas of the colon, stomach, and liver.

DNA methylation is an important epigenetic mechanism for suppressing gene activity by changing the chromatin structure.[4,5] It has become clear that aberrant DNA methylation of promoter region CpG islands may serve as an alternative mechanism to coding region mutation for the inactivation of tumor suppressor or tumor-related genes, and therefore methylation plays an important role in tumorigenesis.[6,7] In biliary tract carcinomas, *E-cadherin*, *p16*, and *RASSF1A* promoter methylation with loss of expression are frequently present, and ultimately result in characteristic biological features,[8–14] whereas *DAP-kinase* (death associated protein kinase) promoter methylation is infrequent in intrahepatic cholangiocarcinomas.[9] However, the roles of other genes, such as *CHFR* and *RUNX3*, which are recently identified tumor suppressor genes silenced by promoter hypermethylation,[15–22] remain to be elucidated. *CHFR* (checkpoint with FHA and RING finger) is a mitotic stress checkpoint gene, whose product mediates a delay of entry into metaphase after treatment with microtubule inhibitors, such as nocodazole or taxol.[15] *RUNX3* (runt-related transcription factor 3), is a major growth regulator of gastric epithelial cells, due to induction of the apoptosis-related action of TGF-β.[16]

In the present study, we investigated the promoter methylation status of tumor suppressor and tumor-related genes, including *CHFR*, *DAP-kinase*, *E-cadherin*, *hMLH1*, *p16*, *RASSF1A*, and *RUNX3*. The clinicopathological significance of gene promoter methylation is discussed.

Materials and Methods

Clinical samples. Surgical specimens were obtained from a total of 37 patients: 23 with bile duct carcinoma (21 extrahepatic and 2 intrahepatic), 9 with gallbladder carcinoma, and 5 with ampullary carcinoma, who underwent surgical resection. Non-neoplastic tissue samples of the extrahepatic bile duct, gallbladder, and ampulla were obtained at autopsy as controls from 2 patients, a 50-year-old male patient who died of cardiac infarction and a 71-year-old male patient who died of pulmonary carcinoma. These tissues were immediately frozen and stored at −80°C until analysis. Fresh-frozen primary biliary tract carcinomas were subjected to genomic DNA extraction.

DNA extraction. Genomic DNA was extracted using a SepaGene Kit (Sanko-Junyaku, Tokyo).

Bisulfite modification and methylation-specific polymerase chain reaction (MSP). Treatment of DNA samples with sodium bisulfite converts all unmethylated cytosines to uracils and does not affect methylated cytosines. Briefly, 2 μg of genomic DNA was denatured with sodium hydroxide and modified with sodium bisulfite. The samples were then purified using Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, recovered in ethanol, and resuspended in 30 μl of distilled water. Amplification was achieved in a 20 μl reaction volume containing 2 μl of GeneAmp PCR Gold Buffer (PE

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Table 1. Clinicopathological characteristics of patients with biliary tract carcinoma

Parameters	Bile duct carcinomas (*n*=23)	Gallbladder carcinomas (*n*=9)	Ampullary carcinomas (*n*=5)	
Age years±SD	69.8±1.4	71.4±1.8	59.2±5.1	*P*=0.04[1)]
Sex (male:female)	11:12	5:4	1:4	NS[2)]
Lymph nodes metastasis	8 (34.8%)	3 (33.3%)	3 (60.0%)	NS[2)]
Stage				NS[2)]
I–II	6 (26.1%)	6 (66.7%)	1 (20.0%)	
III–IV	17 (73.9%)	3 (33.3%)	4 (80.0%)	
Differentiation				NS[2)]
Well	10 (43.5%)	5 (56.6%)	4 (80.0%)	
Moderately	11 (47.8%)	4 (44.4%)	1 (20.0%)	
Poorly	2 (8.7%)	0 (0.0%)	0 (0.0%)	

1) Difference between gallbladder and ampulla of Vater by Mann-Whitney *U* test.
2) Not significant by χ^2 test.

Applied Biosystems, Foster City, CA), 1.0 m*M* $MgCl_2$, 1 μl each primer, 0.2 m*M* dNTPs, and 1 U *Taq* polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems). After heating at 95°C for 10 min, polymerase chain reaction (PCR) was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s, followed by a final 7-min extension at 72°C. A positive control (*Sss* I methylase-treated DNA) and a negative control (distilled water without DNA) were included for each amplification. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. MSP primer sequences were 5′-GGG TCG GAG GGG GTT TTT TC-3′ and 5′-CAA CCG CCG AAC GCA CTC GA-3′ for methylated *p16* (97 bp)[23)]; 5′-ACG TAG ACG TTT TAT TAG GGT CGC-3′ and 5′-CCT CAT CGT AAC TAC CCG CG-3′ for methylated *hMLH1* (115 bp)[24)]; 5′-GTG TTA ACG CGT TGC GTA TC-3′ and 5′-AAC CCC GCG AAC TAA AAA CGA-3′ for methylated *RASSF1A* (93 bp)[25)]; 5′-GGT GAA TTT TTA GTT AAT TAG CGG TAC-3′ and 5′-CAT AAC TAA CCG AAA ACG CCG-3′ for methylated *E-cadherin* (204 bp)[26)]; 5′-ATA ATA GCG GTC GTT AGG GCG TCG-3′ and 5′-GCT TCT ACT TTC CCG CTT CTC GCG-3′ for methylated *RUNX3* (115 bp)[21)]; 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ and 5′-CCC TCC CAA ACG CCC G-3′ for methylated *DAP-kinase* (98 bp)[27)]; 5′-GTA ATG TTT TTT GAT AGC GGC-3′ and 5′-AAT CCC CCT TCG CCG-3′ for methylated *CHFR* (106 bp)[18)]; 5′-GGT TGT AAT GTT TTT TGA TAG TGG T-3′ and 5′-CAA ATC CCC CTT CAC CA-3′ for unmethylated *CHFR* (112 bp)[18)].

Preparation of positive control. *Sss* I methylase (New England BioLabs, Inc., Beverly, MA) was used to methylate 100 μg of peripheral blood DNA, which was modified with sodium bisulfite as described above.

Statistical analysis. Statistical analyses were performed using the χ^2 and Fisher's exact tests for differences between groups, and the Mann-Whitney *U* test for differences between means. Overall survival was calculated using Kaplan-Meier log-rank testing. Cox proportional hazards models were applied for multivariate analysis. A $P<0.05$ was considered significant.

Results

Clinicopathological characteristics. The clinicopathologic characteristics of patients and tumors are summarized in Table 1. Ampullary carcinoma patients were significantly younger than gallbladder carcinoma patients ($P=0.04$). No other significant difference was found.

Promoter methylation status of tumor suppressor and tumor-related genes. The methylation frequencies of *CHFR*, *DAP-kinase*, *E-cadherin*, *hMLH1*, *p16*, *RASSF1A*, and *RUNX3* genes in bile duct carcinomas, gallbladder carcinomas and ampullary carcinomas were 16.2%, 21.6%, 27.0%, 8.1%, 24.3%, 27.0%, and 56.8%, respectively (Fig. 1 and Table 2). Thirty-one (81.8%) of the 37 biliary tract carcinomas exhibited promoter hypermethylation in at least one of these genes. None of the control tissue samples exhibited promoter methylation in any of the gene promoters examined, except that a non-neoplastic tissue sample of the extrahepatic bile duct contained methylated *RASSF1A* promoter. Concurrent methylation of two or more genes occurred in 51.4% (19/37) of the cases. *RUNX3* was more frequently methylated in bile duct carcinomas than in gallbladder carcinomas (78.3% vs. 22.2%; $P=0.006$) and ampullary carcinomas (vs. 20.0%; $P=0.03$). The number of methylated genes per sample was 2.17±0.28 (average±SD) in bile duct carcinomas, 1.80±0.97 in ampullary carcinomas, and 0.89±0.35 in gallbladder carcinomas, with a statistically significant difference between bile duct carcinomas and gallbladder carcinomas ($P=0.02$).

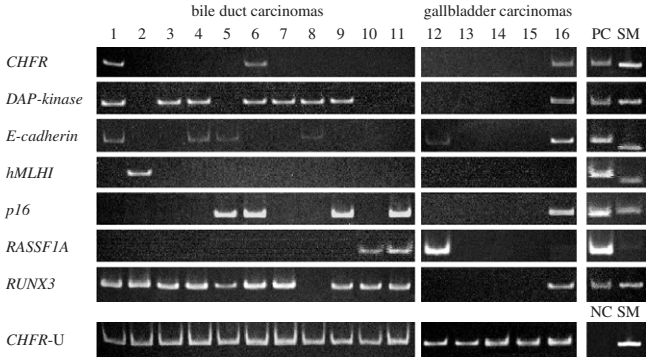

Fig. 1. Examples of methylation-specific PCR (MSP) of bile duct and gallbladder carcinomas. Methylated PCR products are present in lanes 1, 6, and 16 for *CHFR*, in lanes 1, 3, 4, 6–9, and 16 for *DAP-kinase*, in lanes 1, 4, 5, 8, 12, and 16 for *E-cadherin*, in lane 2 for *hMLH1*, in lanes 5, 6, 9, 11, and 16 for p16, in lanes 10, 11, and 12 for *RASSF1A*, and in lanes 1–7, 9–11, and 16 for *RUNX3*. *CHFR*-U indicates unmethylated-sequence-specific PCR for the *CHFR* gene promoter, and unmethylated PCR products are present in all lanes for *CHFR*. Lanes 1–11, bile duct carcinomas; lanes 12–16, gallbladder carcinomas. PC, positive control; NC, negative control; SM, size marker.

Correlation between promoter methylation status and clinicopathological characteristics. The correlations between promoter methylation status and clinicopathological characteristics, in-

Table 2. Frequencies of promoter hypermethylation in biliary tract carcinomas

Primary site	Gene						
	CHFR	*DAP-kinase*	*E-cadherin*	*hMLH1*	*p16*	*RASSF1A*	*RUNX3*
Bile duct carcinomas (*n*=23)	4 (17.4%)	4 (17.4%)	8 (34.8%)	3 (13.0%)	6 (26.1%)	7 (30.4%)	18 (78.3%)
Gallbladder carcinomas (*n*=9)	0 (0.0%)	2 (22.2%)	1 (11.1%)	0 (0.0%)	2 (22.2%)	1 (11.1%)	2 [1] (22.2%)
Ampullary carcinomas (*n*=5)	2 (40.0%)	2 (40.0%)	1 (20.0%)	0 (0.0%)	1 (20.0%)	2 (40.0%)	1 [2] (20.0%)
Total (*n*=37)	6 (16.2%)	8 (21.6%)	10 (27.0%)	3 (8.1%)	9 (24.3%)	10 (27.0%)	21 (56.8%)

1) *P*=0.006 vs. bile duct carcinomas by Fisher's exact test.
2) *P*=0.03 vs. bile duct carcinomas by Fisher's exact test.

Table 3. Correlation between promoter methylation status and clinicopathological characteristics in biliary tract carcinomas

Gene	Methylation status		Age (mean±SD)[2]		Lymph node metastasis[4]			Stage[4]			Differentiation[4]		
					Positive	Negative		I–II	III–IV		well-moderately	poorly	
CHFR	M[1]	6	63.3±3.5	NS[3]	2	4	NS	1	5	NS	5	1	NS
	U	31	69.8±1.4		12	19		12	19		30	1	
DAP-kinase	M	8	69.6±2.9	NS	3	5	NS	2	6	NS	6	2	*P*=0.04
	U	29	68.6±1.5		11	18		11	18		29	0	
E-cadherin	M	10	69.1±2.4	NS	2	8	NS	5	5	NS	9	1	NS
	U	27	68.7±1.6		12	15		8	19		26	1	
hMLH1	M	3	67.0±6.0	NS	0	3	NS	1	2	NS	3	0	NS
	U	34	68.9±1.4		14	20		12	22		32	2	
p16	M	9	71.0±2.0	NS	3	6	NS	4	5	NS	8	1	NS
	U	28	68.1±1.6		11	17		9	19		27	1	
RASSF1A	M	10	69.9±2.3	NS	3	7	NS	3	7	NS	10	0	NS
	U	27	68.4±1.6		11	16		10	17		25	2	
RUNX3	M	21	71.8±1.2	*P*=0.01	10	11	NS	6	15	NS	19	2	NS
	U	16	64.8±2.3		4	12		7	9		16	0	

1) M, methylated; U, unmethylated.
2) Comparison was between methylated and unmethylated by Mann-Whitney *U* test.
3) Not significant.
4) Comparison was made by Fisher's exact test.

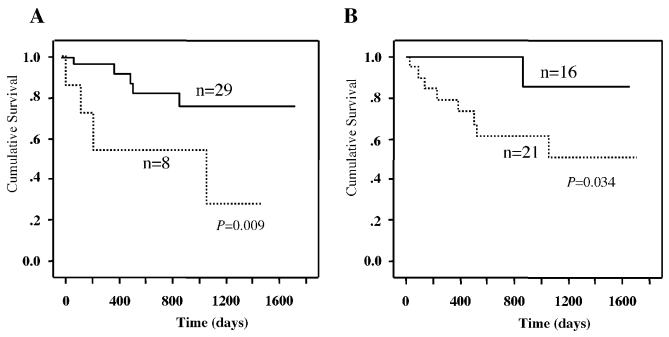

Fig. 2. Overall survival of biliary tract carcinoma patients according to promoter methylation status of *DAP-kinase* (A) and *RUNX3* (B) genes. Solid line, patients with an unmethylated gene promoter; dotted line, patients with a methylated gene promoter.

cluding the patient's age, lymph node metastasis, stage, and tumor differentiation, are shown in Table 3. The patients with methylated *RUNX3* were significantly older than those with an unmethylated *RUNX3* promoter (*P*=0.01). *DAP-kinase* methylation was more frequently detected in poorly differentiated tumors than in well to moderately differentiated ones (*P*=0.04). No other significant correlation was found.

Promoter methylation status and patients' prognoses. The patients with methylated promoter of *RUNX3* or *DAP-kinase* showed significantly shorter survival times than those with an unmethylated promoter (Fig. 2). No significant correlation was detected for the other genes. In a multivariate analysis model that included the methylation status of each gene, sex and age of patients, lymph node metastasis, and tumor stage, *DAP-kinase* methylation-positive status was an independent prognostic factor (hazard ratio=8.71, *P*=0.024).

Discussion

In this study, 37 samples of primary biliary tract carcinoma were analyzed for methylation status of tumor suppressor and tumor-related genes by MSP. Thirty-one (81.8%) of the 37 biliary tract carcinomas exhibited promoter hypermethylation in at least one of these genes. Concurrent methylation of two or more genes occurred in 51.4% (19/37) of the cases. These results indicate that there is widespread methylation of gene promoters in a subset of biliary tract carcinomas. Corresponding non-neoplastic tissue samples were not available in this study, so we studied non-neoplastic tissue samples obtained at autopsy, and found that promoter methylation was exceptional in these materials. Further analysis is necessary to clarify age-related methylation of biliary tract epithelia, although the absence of methylation of 18 tumor suppressor and tumor-related genes in 15 normal bile duct samples has been reported.[9]

The *p16* gene plays an important role in controlling the cell cycle,[28] *hMLH1* is active in mismatch repair,[29] *DAP-kinase* is a

positive mediator of apoptosis induced by IFN-γ,[30] and *E-cadherin* is a cell-cell adhesion molecule,[31] and thus these genes are important in human tumorigenesis. Methylation of these genes has been found in a variety of human tumor types including gastric, colorectal, lung, ovarian, breast, and hepatocellular carcinomas.[7, 32, 33] The *RASSF1A* gene, known as an isoform gene encoding the Ras effector, is also inactivated epigenetically in many tumor types.[25, 34–40] Frequent methylation of these genes has also been reported in biliary tract carcinomas.[8–14] For example, *p16* methylation was detected in 22.2%–42.9% of extrahepatic bile duct carcinomas,[8, 12] 17.7%–83.0% of intrahepatic cholangiocarcinomas,[9, 12] 56.0% of gallbladder carcinomas,[14] and 22.2% of ampullary carcinomas[8]; *hMLH1* in 0% of extrahepatic bile duct carcinomas,[8] 14.0% of gallbladder carcinomas,[14] and 11.1% of ampullary carcinomas[8]; *DAP-kinase* in 7.6% of intrahepatic cholangiocarcinomas[9]; *E-cadherin* in 21.5% of intrahepatic cholangiocarcinomas[9]; and *RASSF1A* in 69.2% of intrahepatic cholangiocarcinomas.[10] Methylation frequencies of these genes in the present study were found to be mostly comparable to those found in previous studies. We also found that the number of methylated genes per sample was significantly more in bile duct carcinomas than in gallbladder carcinomas. Promoter methylation is site-specific for each gene, and bile duct carcinomas are more prone to be affected by promoter methylation when compared with gallbladder carcinomas.

There have been no reports yet concerning the methylation status of *CHFR* and *RUNX3* in biliary tract carcinomas. The frequency of *CHFR* methylation was 16.2% in biliary tract carcinomas in our present study. This result is similar to those in esophageal and lung carcinomas.[18, 19] *CHFR* may induce chromosomal instability due to delayed chromosome condensation[41] and resultant DNA aneuploidy. The low frequency of *CHFR* methylation despite the very high frequency of DNA aneuploidy in biliary tract carcinomas[42] may indicate that other genes play more important roles in the evolution of aneuploid clones. *RUNX3* was found to be methylated in 56.8% of biliary tract carcinomas, and methylation was more frequent in bile duct carcinomas than in gallbladder and ampullary carcinomas. In the *RUNX3* knock-out mouse, the gastric mucosa exhibits hyperplasia due to stimulated proliferation and suppressed apoptosis of epithelial cells, and the cells are resistant to the growth-inhibitory and apoptosis-inducing action of TGF-β, indicating that *RUNX3* is a major growth regulator of gastric epithelial cells.[16] There have been no reports concerning the role of *RUNX3* in biliary tract epithelium; however, *RUNX3* may also regulate proliferation of biliary tract epithelium, and its silencing by epigenetic alteration may play an important role in biliary tract carcinogenesis. The significant difference of *RUNX3* methylation frequencies between carcinomas of bile duct and those of gallbladder and ampulla Vater may suggest the existence of compounds that accelerate promoter methylation in bile duct epithelia.

Age-related methylation may have the potential to lead to carcinogenesis.[23, 43–47] In the present study, methylation of *RUNX3* was more frequent in elderly patients, whereas there was no significant correlation between methylation status and aging for the other genes examined. Environmental factors such as inflammation,[48] tobacco smoking[49] and *Helicobacter pylori* infection,[50] can accelerate DNA methylation. It is possible that certain environmental factors act more directly on bile duct epithelia than on epithelial cells of the gallbladder and ampulla. Because methylation of *DAP-kinase* was more frequent in poorly differentiated tumors than in well to moderately differentiated ones, as also found in studies of gastric[21] and hepatocellular carcinomas,[51] *DAP-kinase* methylation might be associated with tumor differentiation in many types of human tumors. The overall survival rate was significantly lower in patients with methylated *DAP-kinase* or *RUNX3* compared to those with unmethylated genes. Furthermore, *DAP-kinase* methylation-positive status was independently associated with poor survival in multivariate analyses. *DAP-kinase* promoter methylation was also associated with a worse prognosis in patients with non-small cell lung cancer[52] or multiple myeloma.[53] There have been no reports about the prognostic value of *RUNX3* methylation. It is interesting that hypermethylation of *DAP-kinase* and *RUNX3*, which leads to uncontrolled cell death through disruption of different apoptotic pathways, affects the prognosis of patients with biliary tract carcinomas. It is difficult to assess gene silencing in surgical specimens because tumor specimens usually contain not only tumor cells, but also several kinds of non-tumor cells, such as non-neoplastic epithelia, inflammatory cells, and other stromal cells, and therefore mRNA might be detected even though critical promoter regions are methylated in tumor cells. In addition, methylation does not necessarily cause gene silencing.[54] As for *DAP-kinase* and *RUNX3*, whose methylation was associated with poor survival, the regions analyzed in the present study have been shown to be associated with gene silencing.[21, 27] Therefore, detection of methylated genes is more practical than that of gene silencing for estimation of patients' prognoses.

In conclusion, a significant proportion of primary biliary tract carcinomas exhibited promoter hypermethylation of tumor suppressor and tumor-related genes, although bile duct carcinomas are more prone to being affected by promoter methylation than are gallbladder carcinomas. Hypermethylation of *DAP-kinase* has been found to be a significant prognostic factor in primary biliary tract carcinomas.

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