Increased DNA methyltransferase 1 (DNMT1) protein expression in precancerous conditions and ductal carcinomas of the pancreas

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Aberrant DNA methylation has been shown to play an important role during multistage carcinogenesis in various human organs. The aim of the present study was to evaluate the significance of DNA methyltransferase 1 (DNMT1) protein expression during pancreatic carcinogenesis. Immunohistochemical analysis of DNMT1 in 48 peripheral pancreatic duct epithelia showing no remarkable histological findings without an inflammatory background (DE), 54 peripheral pancreatic duct epithelia with an inflammatory background (DEI), 188 pancreatic intraepithelial neoplasias (PanIN), and 220 areas of invasive ductal carcinoma from surgical specimens resected from 100 patients, was carried out. The average incidence of DNMT1 immunoreactivity increased progressively from DE to DEI ($P = 0.003$), from DE and DEI to PanIN ($P < 0.0001$), among PanIN with different grades of dysplasia (from PanIN I to PanIN II, $P = 0.0012$), from PanIN to invasive ductal carcinomas ($P < 0.0001$) and among invasive ductal carcinomas with different grades of histological differentiation (from well or moderately to poorly differentiated adenocarcinomas, $P < 0.0001$). High-level DNMT1 protein expression in invasive ductal carcinomas was correlated significantly with an advanced t category ($P = 0.0224$) and an advanced stage ($P = 0.0294$). Moreover, patients with invasive ductal carcinomas showing high-level DNMT1 protein expression had a poorer outcome ($P = 0.0469$). These data suggest that increased DNMT1 protein expression participates in multistage pancreatic carcinogenesis from the precancerous stage to malignant progression of ductal carcinomas and may be a biological predictor of poor prognosis. (*Cancer Sci* 2005; 96: 403–408)

Pancreatic cancer is a devastating disease with a very poor prognosis, with a 5-year survival rate of < 3%, and is the fourth or fifth largest cause of cancer-related death worldwide.[1,2] Because ductal carcinomas frequently emerge in pancreases damaged by chronic pancreatitis,[3] at least a proportion of peripheral pancreatic duct epithelia with an inflammatory background may be at the precancerous stage, even though they may show no remarkable histological findings. Recently, Hruban *et al*.[4] suggested a new nomenclature and classification system for pancreatic intraepithelial neoplasia (PanIN) as a precancerous lesion, and proposed a model of progression from PanIN to ductal carcinoma.[5] Elucidation of genetic and epigenetic alterations in such precancerous conditions and ductal carcinomas showing various clinicopathological features would contribute to a better understanding of the molecular basis of multistage pancreatic carcinogenesis.

DNA methylation plays an important role in transcriptional regulation, chromatin remodeling and genomic stability.[6] Overall DNA hypomethylation and regional DNA hypermethylation are commonly observed in various tumors, including pancreatic cancers.[7,8] Furthermore, accumulating evidence suggests that aberrant DNA methylation is involved even in the early and precancerous stages of human carcinogenesis.[9–15]

DNA methyltransferase 1 (DNMT1) is the major human DNMT[16] and increased levels of its mRNA and protein expression have been reported in several human precancerous conditions and cancers.[17–22] We have reported previously that DNMT1 protein overexpression precedes an increase in the proliferating cell nuclear antigen labeling index in precancerous conditions of the urinary bladder,[21] and is significantly correlated with poorer differentiation of liver[19] and stomach[22] cancers and a poor prognosis in patients with liver cancer.[19] However, to our knowledge, there are no reported data on the expression of DNMT1 at both the mRNA and protein levels in pancreatic cancers. In this study we carried out an immunohistochemical analysis of DNMT1 expression in a large series of precancerous conditions and ductal carcinomas of the pancreas to evaluate its significance in multistage pancreatic carcinogenesis.

Materials and Methods

Patients and samples. A total of 48 peripheral pancreatic duct epithelia showing no remarkable histological findings without an inflammatory background (DE), 54 peripheral pancreatic duct epithelia with an inflammatory background (DEI, such ducts were surrounded by infiltrating lymphocytes) and 188 pancreatic intraepithelial neoplasias (PanIN; 50 PanIN IA, 126 PanIN IB and 12 PanIN II) were obtained from surgical specimens resected from 100 patients at the National Cancer Center Hospital, Tokyo, between 1997 and 2002. Invasive ductal carcinomas from this cohort frequently showed histological heterogeneity (e.g. well, moderately or poorly differentiated adenocarcinoma components were simultaneously observed even in tissue sections from any single patient). Then 220 areas of invasive ductal carcinoma (58 well-differentiated adenocarcinomas [WD], 114 moderately differentiated adenocarcinomas [MD], and 48 poorly differentiated adenocarcinomas [PD]) were examined from these 100 patients. The patients comprised 56 men and 44 women with a mean age ± SD of 62.26 ± 10.17 years (range, 33–83 years). Histopathological evaluation of the PanIN and cancers was carried out by three pathologists (Dun-Fa Peng, Yae Kanai, and Nobuyoshi Hiraoka), according to previously published criteria.[4,23] The study was approved by the Ethics Committee of the National Cancer Center, Tokyo.

Immunohistochemistry. Three-micrometer-thick sections of formalin-fixed, paraffin-embedded tissue specimens were deparaffinized and dehydrated. After antigen retrieval by heating

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in an autoclave for 10 min at 120°C, the sections were incubated with 2% normal swine serum to block any non-specific reaction. Then the sections were incubated with a goat antihuman polyclonal antibody against DNMT1 (N16, dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by incubation with a biotinylated secondary antibody (antigoat IgG, 1:200; Vector Laboratories, Burlingame, CA) and Vectastain Elite ABC reagent (Vector Laboratories) at room temperature for 30 min each. We had previously confirmed the specificity of the goat antihuman DNMT1 polyclonal antibody by western blotting analysis: an immunoreactive band of approximately 193.5 kDa, corresponding to the molecular mass of DNMT1, was detected in human cancer cells, but no non-specific bands were detected with this antibody.[19] The sections were then treated with 3,3′-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin. Tissue specimens of stomach cancers, in which we had detected positive immunoreactivity for DNMT1 in our previous study,[22] were used as a positive control at each staining. As described previously,[21,24] lymphocytes on the same slide were used as an internal positive control for DNMT1 immunoreactivity.

For each sample ($n = 510$), at least 500 cells were randomly counted. If the lesion was small with less than 500 cells, all the cells were counted. In all samples, DNMT1 immunoreactivity was detected only in the nucleus and never in the cytoplasm and cell membrane. The incidence of DNMT1 immunoreactivity in each sample was expressed as a percentage of all the cells counted. For each patient ($n = 100$), the level of DNMT1 protein expression in the cancer was considered to be low if less than 20% of cancer cells showed DNMT1 immunoreactivity, and high if 20% or more of the cancer cells were positive for DNMT1 after a thorough evaluation of two or three representative tissue sections that frequently showed histological heterogeneity and simultaneously contained well, moderately or poorly differentiated adenocarcinoma components.

Statistics. Comparisons of the incidence of DNMT1 immunoreactivity between or among sample groups (DE, DEI, PanIN, WD, MD, and PD) were analyzed by the Mann–Whitney U or Kruskal–Wallis test. Correlations between the level of DNMT1 protein expression and clinicopathological parameters were analyzed by the χ^2 test. Survival curves were calculated with the Kaplan–Meier method and the significance was analyzed by log–rank test. For all tests, $P < 0.05$ was considered to be the level of significance.

Results

Incidence of DNMT1 immunoreactivity in precancerous conditions and ductal carcinomas of the pancreas. Figures 1 and 2 show examples of immunohistochemical staining for DNMT1 in DE, DEI, PanIN and invasive ductal carcinomas of the pancreas. Figure 3 summarizes the results of immunohistochemistry for DNMT1. There was a significant difference in the average incidence of DNMT1 immunoreactivity between DE and DEI ($P = 0.0003$, Figs 1a,b and 3), and between ductal epithelia showing no remarkable histological findings (DE and DEI) and

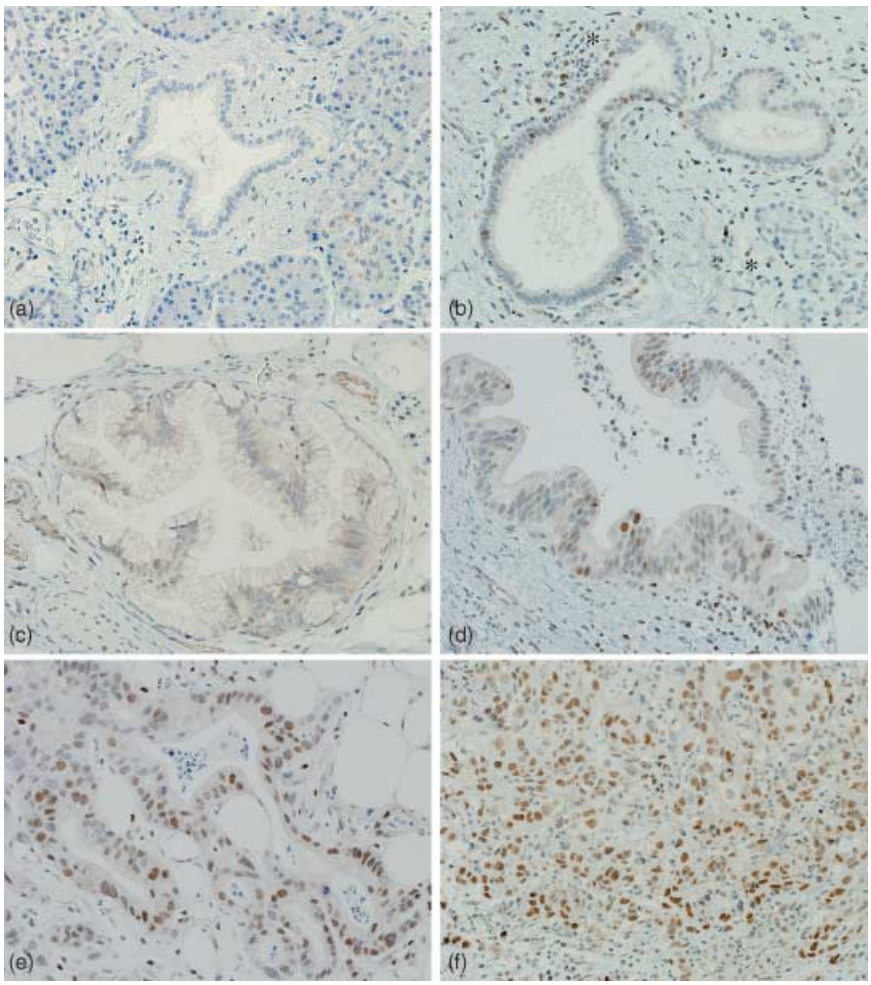

Fig. 1. Immunohistochemical examination for DNA methyltransferase 1 (DNMT1) in tissue specimens. No detectable DNMT1 immunoreactivity was observed in peripheral pancreatic duct epithelia showing no remarkable histological findings without an inflammatory background (a). However, scattered DNMT1-positive cells were observed in peripheral duct epithelia with an inflammatory background accompanied by pancreatic acinar atrophy (b). In panel (b), infiltrating lymphocytes as internal controls show positive immunoreactivity for DNMT1 (*). The incidence of DNMT1 immunoreactivity increased progressively from pancreatic intraepithelial neoplasia (PanIN) IB (c), PanIN II (d), moderately differentiated adenocarcinoma (e), to poorly differentiated adenocarcinoma (f). ×200

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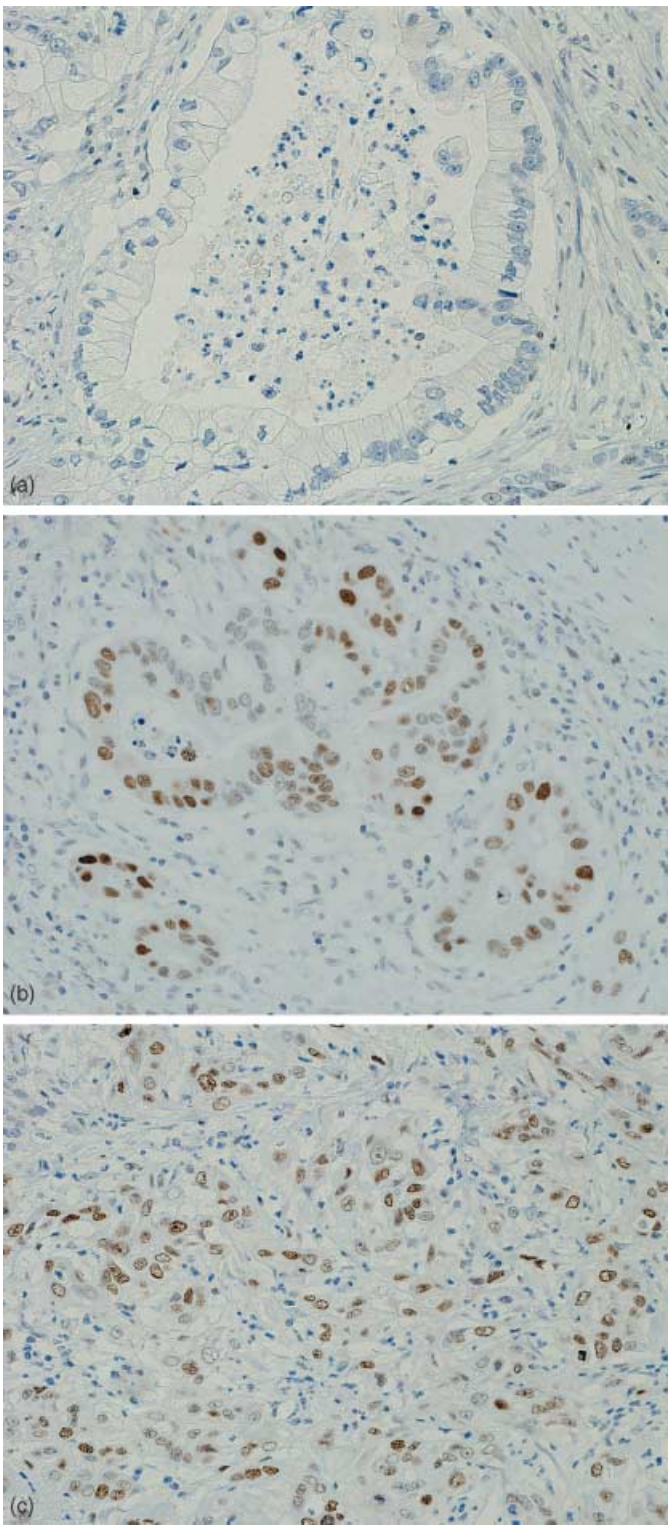

Fig. 2. Heterogeneity of DNA methyltransferase 1 (DNMT1) protein expression among components showing different grades of histological differentiation in a representative cancer from a single patient. Moderately (b) and poorly (c) differentiated adenocarcinoma components showed a higher incidence of DNMT1 immunoreactivity than the well-differentiated adenocarcinoma component (a).

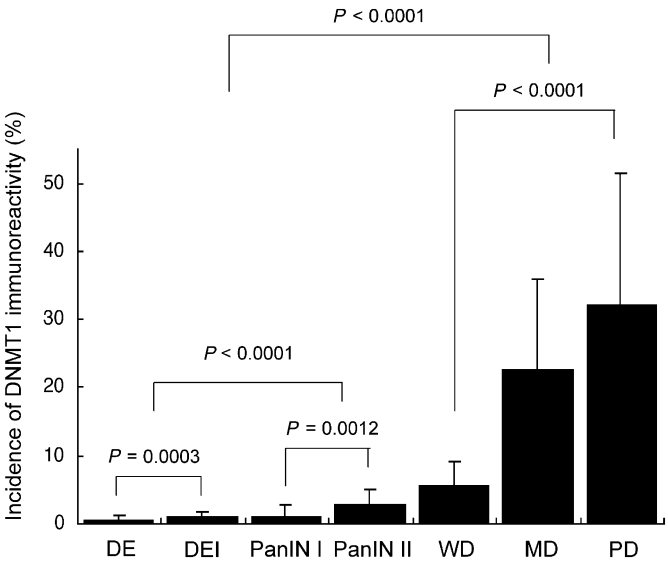

Fig. 3. A summary of the incidence of DNA methyltransferase 1 (DNMT1) immunoreactivity in precancerous conditions and ductal carcinomas of the pancreas. Immunohistochemical examination was performed. For each sample (n = 510) at least 500 cells were randomly counted. The incidence of DNMT1 immunoreactivity in each sample was expressed as a percentage of all the cells counted. A progressive increase in the incidence of DNMT1 immunoreactivity was observed during multistage pancreatic carcinogenesis. The error bars represent standard deviation. DE, peripheral pancreatic duct epithelia showing no remarkable histological findings without an inflammatory background; DEI, peripheral pancreatic duct epithelia with an inflammatory background; PanIN, pancreatic intraepithelial neoplasia; WD, well-differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma.

PanIN (PanIN I and PanIN II) ($P < 0.0001$, Fig. 3). A significant difference between PanIN I and PanIN II was detected ($P = 0.0012$, Figs 1c,d and 3), although no PanIN III lesion was ever identified in the non-cancerous pancreatic tissue from the examined tissue sections of all 100 patients. Moreover, there was a significant difference between all PanIN (PanIN I and PanIN II) and all invasive ductal carcinomas (WD, MD and PD) ($P < 0.0001$, Fig. 3), and a progressive increase in the incidence of DNMT1 immunoreactivity was observed among WD, MD and PD ($P < 0.0001$, Figs 1e,f and 3). With respect to the histological heterogeneity observed even in the lesion from a single patient, the incidence of DNMT1 immunoreactivity showed a tendency to increase progressively from its well, moderately to poorly differentiated adenocarcinoma components (Fig. 2).

Correlations between DNMT1 protein expression level and clinicopathological parameters in invasive ductal carcinomas of the pancreas. Correlations between the level of DNMT1 protein expression (low-level or high-level) and clinicopathological parameters[23] in invasive ductal carcinomas from all 100 patients (n = 100) are summarized in Table 1. High-level DNMT1 protein expression in invasive ductal carcinomas was significantly correlated with an advanced t category defined on the basis of the extent of cancer invasion to the anterior pancreatic capsule, retroperitoneal tissue, bile duct, duodenal wall, portal venous system and arterial system[23] and an advanced stage ($P = 0.0224$ and $P = 0.0294$, respectively, Table 1). No significant correlation was observed between the level of DNMT1 protein expression and patient age, sex, and other clinicopathological parameters (Table 1).

Correlation between DNMT1 protein expression level and prognosis of pancreatic cancer patients. We examined the prognostic impact of DNMT1 protein expression in 74 patients who underwent curative resection by partial pancreatoduodenectomy or distal pancreatectomy. The overall survival curve of the 74

Table 1. Correlation between DNMT1 protein expression level and clinicopathological parameters

Clinicopathological parameters*	DNMT1 expression		*P*-value
	Low-level	High-level	
t Category			0.0224
t1	5	0	
t2	22	11	
t3	29	33	
Lymph node involvement			0.0633
negative	13	4	
positive	42	41	
Staging			0.0294
I + II	5	0	
III	16	8	
IVa	21	28	
IVb	14	8	
Invasion pattern			0.2859
α	0	0	
β	30	27	
γ	26	15	
Lymphatic involvement			0.4972
0	4	2	
1	21	14	
2	28	22	
3	3	6	
Venous involvement			0.2197
0	7	1	
1	14	13	
2	30	23	
3	5	7	
Intrapancreatic nerve involvement			0.2907
0	3	2	
1	18	12	
2	21	24	
3	13	5	
Sex			0.2335
Male	29	28	
Female	27	16	
Age (mean ± SD)	62.63 ± 10.13	61.80 ± 10.33	0.6878

DNMT1, DNA methyltransferase 1. *Evaluation of clinicopathological parameters was carried out according to reference (23).

patients with high- and low-level DNMT1 protein expression was calculated by the Kaplan–Meier method (Fig. 4). A significant difference was observed between the groups with high and low DNMT1 protein expression ($P = 0.0469$, log rank test).

Discussion

We believe that this is the first report to describe the expression of DNMT1 protein in precancerous conditions and ductal cancers of the pancreas. Initially, the incidence of DNMT1 immunoreactivity increased in DEI compared to DE. Inflammatory cell infiltration around the peripheral pancreatic duct was frequently associated with pancreatic acinar atrophy, suggesting that persistent inflammation had long been present in such areas. Although ductal carcinomas are not always preceded by chronic pancreatitis, and the persistent inflammation observed in a part of our cohort may have been induced secondarily by obstruction of the pancreatic ducts as a result of tumor growth, it is well known that chronic pancreatitis significantly increases the risk of developing pancreatic cancer.[3] Therefore, at least a proportion of DEI may be at the precancerous stage even though they may show no remarkable histological findings. Our data suggest that increased expression of DNMT1 protein may participate even in the very early stage of multistage pancreatic carcinogenesis. It has been reported that some pancreatic ducts showing no remarkable histological findings

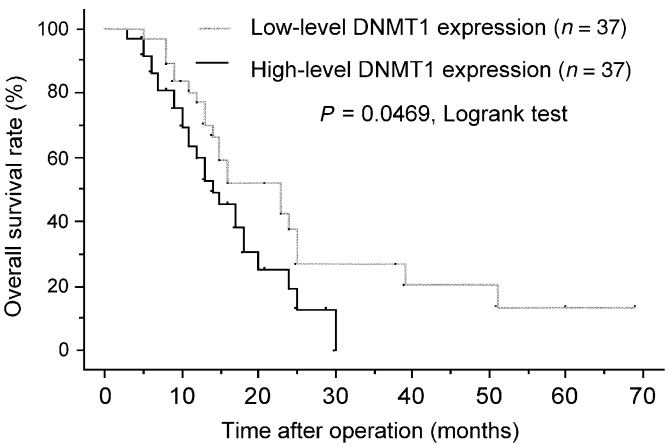

Fig. 4. Kaplan–Meier survival curve. Immunohistochemical examination was performed. For each patient who underwent curative resection by partial pancreatoduodenectomy or distal pancreatectomy ($n = 74$), the level of DNA methyltransferase 1 (DNMT1) protein expression in the cancer was considered to be low if less than 20% of the cancer cells showed DNMT1 immunoreactivity, and high if 20% or more of the cancer cells were positive for DNMT1 after a thorough evaluation of two or three representative tissue sections. The patients with a high level of DNMT1 protein expression ($n = 37$) had a poorer prognosis than those with a low level ($n = 37$) ($P = 0.0469$, log rank test).

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have K-ras mutation[25] and loss of heterozygosity on some loci.[26] In combination with such genetic aberrations, increased DNMT1 protein expression could play a role in triggering the process of tumorigenesis in ductal epithelia with an inflammatory background. Further studies are needed to understand the molecular mechanisms by which an inflammatory microenvironment affects the level of DNMT1 expression in ductal epithelia.

The incidence of DNMT1 immunoreactivity increased progressively in PanIN compared to DE and DEI and among PanIN that were accompanied by increasing grades of dysplasia, and was further increased in invasive ductal carcinomas, clearly indicating a continuous association of DNMT1 protein overexpression with the progression of multistage pancreatic carcinogenesis. In stomach and colorectal cancers, we have reported that DNMT1 overexpression results in a CpG island methylator phenotype,[20,22] which is defined as frequent DNA hypermethylation on CpG islands that are not methylated in normal tissues.[27] DNA hypermethylation on CpG islands of several cancer-related genes has been reported in PanIN and ductal cancers of the pancreas.[28] By analogy with the findings for stomach and colorectal cancers, DNMT1 protein overexpression may actually result in DNA hypermethylation on such CpG islands of cancer-related genes during pancreatic carcinogenesis. As DNMT1 shows a preference for hemimethylated rather than unmethylated substrates *in vitro*,[29] and targets replication foci by binding to proliferating cell nuclear antigen,[30] it has been considered to be a maintenance form of DNMT that copies methylation patterns after DNA replication. However, in human cancers, unknown factors may potently target DNMT1 to unmethylated substrate DNA, such as CpG islands of specific genes. Moreover, some workers have proposed that DNMT1 possesses both maintenance and *de novo* DNA methylation activity *in vivo*, regardless of its *in vitro* substrate preference.[31] Therefore, it is feasible that DNMT1 protein overexpression actually results in *de novo* and regional DNA hypermethylation during carcinogenesis.

In the present study, we noted that the incidence of DNMT1 immunoreactivity increased abruptly from WD to MD and was further increased in PD (Fig. 3). Even in the same patient, PD components showed a much higher incidence of DNMT1 immunoreactivity than WD or MD components (Fig. 2). Accordingly, we detected a significant correlation between high-level DNMT1 protein expression and an advanced t category and advanced stage at diagnosis defined by the Japan Pancreatic Society.[23] However, high-level DNMT1 protein expression did not show a significant correlation with the clinicopathological parameters defined by the UICC classification.[32] This discrepancy may be attributable to the fact that the classification we used[23] reflects in detail the extent of the tumor by using more parameters than the UICC classification.[32] Our data suggest that DNMT1 protein overexpression was associated with aggressiveness of pancreatic cancers. We then analyzed the overall survival curve by the Kaplan–Meier method (Fig. 4). Almost half of the patients died within the first year after surgery, irrespective of their DNMT1 protein expression level. This appears to reflect the devastating nature of pancreatic cancer. However, a significant difference was observed between the patient groups with a high- and a low-level of DNMT1 protein expression: those with high-level DNMT1 protein expression had a worse prognosis from approximately the second year after surgery. Immunohistochemical examination of DNMT1 in cytological, biopsy or surgically resected specimens may be clinically useful for predicting the outcome of patients with pancreatic cancer.

Although DNMT1 is the major DNMT in humans, the activity of DNMT3a and DNMT3b has also been confirmed.[33] DNA methylation patterns are considered to be established through the cooperation of the three DNMT. Further immunohistochemical studies on the cooperative action of DNMT1 with other DNMT in tissue specimens may provide further understanding of the background factors determining the DNA methylation pattern in pancreatic cancers. DNMT may become a molecular target for preventive and therapeutic strategies against multistage pancreatic carcinogenesis.

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