DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas

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To evaluate the significance of alterations in DNA methylation during multistage carcinogenesis of the pancreas, tissue samples of 13 peripheral pancreatic duct epithelia showing no remarkable histological changes without inflammatory background (DE), 20 peripheral pancreatic duct epithelia showing no remarkable histological changes with inflammatory background (DEI), 40 pancreatic intraepithelial neoplasias (PanIN) and 147 areas of ductal carcinoma were microdissected from surgically resected specimens from 58 patients and were embedded into agarose beads. The embedded tissue samples were subjected to methylation-specific PCR (MSP) to evaluate the DNA methylation status of the p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1 and DAPK-1 genes. The prevalence of DNA methylation of at least one of the 12 genes and the average number of methylated genes were significantly higher in both DEI (60% and 0.85 ± 0.88, P = 0.0151 and P = 0.0224, respectively) and PanIN (67.5% and 0.95 ± 0.85, P = 0.0014 and P = 0.0028, respectively) than in DE (15.4% and 0.15 ± 0.38), and were further increased in ductal carcinoma (98.3% and 2.50 ± 1.35, P < 0.0001 and P < 0.0001, respectively). The BRCA1, APC, p16 and TIMP-3 genes were frequently methylated in ductal carcinoma (60.3, 58.6, 39.3 and 30.9%, respectively). Considerable heterogeneity of DNA methylation status was observed among multiple microdissected areas from individual ductal carcinomas, and the number of methylated genes per area was significantly correlated with poorer tumor differentiation (P = 0.0249). The average number of methylated genes in ductal carcinomas was significantly correlated with DNMT1 protein expression level (P = 0.0093). These data suggest that accumulation of DNA methylation of multiple tumor-related genes is involved in multistage carcinogenesis of the pancreas from early precancerous stages to malignant progression and that DNMT1 protein overexpression may be responsible for this aberrant DNA methylation.

Introduction

Accumulating evidence demonstrates that carcinoma in various organs is associated with aberrant DNA methylation, namely overall DNA hypomethylation and regional DNA hypermethylation (1–5). Deamination of 5-methylcytosine to thymine increases gene mutagenicity, and aberrant DNA methylation is associated with chromosomal instability (1). Moreover, DNA methylation of CpG islands located in the promotor regions of tumor-related genes, such as p14, p15, p16, p73, APC, hMLH1, BRCA1, MGMT, GSTP1, CDH1, TIMP-3 and DAPK-1, is associated with silencing of such genes (6). Our group has reported that overexpression of DNA methyltransferase 1 (DNMT1), a major DNA methyltransferase, is significantly correlated with accumulation of DNA methylation of tumor-related genes and/or C-type CpG islands, which are known to be methylated in a cancer-specific but not an age-dependent manner (7), in carcinomas derived from various organs (8–11).

Pancreatic carcinoma is one of the most lethal human carcinomas (12). Ductal carcinoma of the pancreas is believed to originate from peripheral pancreatic duct epithelia, and chronic pancreatitis is regarded as one of the risk factors for the development of pancreatic carcinoma (13,14). Thus, at least part of peripheral pancreatic duct epithelia with inflammatory background may be at a precancerous stage. Pancreatic intraepithelial neoplasia (PanIN) is regarded as a precancerous lesion of ductal carcinoma (15,16). Although a few reports on the DNA methylation status of ductal carcinoma of the pancreas are available (17–19), such information has never been reported for peripheral pancreatic duct epithelia without or with inflammatory background and therefore we are unable to compare DNA methylation status in ductal carcinoma with that of the exact tissue of origin or of the precancerous stage of the tumor. In addition, rather little epigenetic information is available for PanIN (20). Consequently, the significance of alterations in DNA methylation during multistage carcinogenesis of the pancreas is not fully understood. We recently reported that DNMT1 protein expression increased progressively during the stages of pancreatic carcinogenesis (i.e. peripheral pancreatic duct epithelia showing no remarkable histological changes without inflammatory background (DE), peripheral pancreatic duct epithelia showing no remarkable histological changes with inflammatory background (DEI), PanIN and ductal carcinoma) and was associated with tumor aggressiveness, suggesting that protein overexpression of DNMT1 may be involved in multistage carcinogenesis of the pancreas from early stages to malignant progression (21). However, the correlation between accumulation of DNA methylation of tumor-related genes and DNMT1 overexpression has never been examined in ductal carcinoma of the pancreas.

To improve our understanding of the significance of alterations in DNA methylation during multistage carcinogenesis...
of the pancreas, we evaluated the DNA methylation status of 12 tumor-related genes in DE, DEI, PanIN and ductal carcinoma and examined the correlation between DNA methylation status of these tumor-related genes and the protein expression levels of DNMT1 in the same tissue samples.

Materials and methods

Patients and tissue samples

In total, 220 tissue samples, including 13 samples of DE, 20 samples of DEI, 40 samples of PanIN (4 samples of PanIN IA, 28 samples of PanIN IB, 8 samples of PanIN II) and 147 areas of ductal carcinoma, were microdissected from surgically resected specimens from 58 patients. These patients had undergone duodenopancreatectomy or pancreatosplenectomy for invasive ductal carcinoma of the pancreas at the National Cancer Center Hospital, Tokyo, Japan, and comprised 30 males and 28 females aged from 42 to 83 years (mean 61.45 ± 9.67 years). Histopathological evaluation of the PanINs and carcinomas was carried out by three pathologists (Peng DF, Kanai Y and Hiraoka N) according to previously published criteria (15,22). HE-stained formalin-fixed paraffin-embedded tissue slides, 10 μm thick, were subjected to microdissection with a surgical blade under a stereoscopic microscope. After microdissection, high-purity samples of DE, DEI, PanIN and ductal carcinoma could easily be harvested, avoiding contamination with each other and with acinar cells, lymphocytes and abundant desmoplastic stroma (Figure 1). Because ductal carcinomas frequently showed histological heterogeneity (well, moderately or poorly differentiated adenocarcinoma components were present in tissue sections from an individual patient), 1–5 areas (about 500–1000 cancer cells/area) were microdissected from the individual carcinomas to evaluate the heterogeneity of DNA methylation status. Consequently, 147 areas were microdissected from the 58 ductal carcinomas.

The study was approved by the Ethics Committee of the National Cancer Center, Tokyo.

Analysis of DNA methylation status

Agarose beads-based bisulfite modification was performed as described previously, with modification (23,24). Briefly, preheated low-melting agarose (SeaPlaque agarose; BMA, Rockland, ME, USA) was mixed with the harvested tissue samples and the mixtures were pipetted into chilled mineral oil (Sigma-Aldrich, St Louis, MO, USA) to form agarose beads. The beads with tissue were incubated in lysis solution (10 mM Tris–HCl, 10 mM EDTA, 1% SDS, 20 μg/ml proteinase K) for 48 h at 50°C and then treated at room temperature with 0.3 N NaOH for 2 × 15 min and 0.1 N NaOH for 5 min. The individual beads were then heated to 80°C for 15 min to fully separate individual DNA strands in the liquid agarose. The DNA-agarose solutions were allowed to re-solidify on ice and the beads were treated with bisulfite solution (2.5 M NaOH for 2 × 15 min and 15.5 M hydroiodic acid, pH 5.0) for 4 h at 50°C, washed with TE (10 mM Tris–HCl and 1 mM EDTA) for 6 × 15 min and desulfonated in 0.2 N NaOH for 2 × 15 min. After neutralization with hydrochloric acid, the beads were used directly for nested methylation-specific PCR (MSP). DNA methylation status on the promoter regions of the 12 examined tumor-related genes in tissue samples from 58 patients was evaluated with the primers described previously (25). Products of the first PCR with primer sets designed to amplify both methylated and unmethylated genomic regions equally were diluted 1 : 100 and subjected to the second PCR with primer sets for each gene designed to recognize bisulfite-induced sequence differences between methylated and unmethylated genomic regions. Universally methylated DNA and unmethylated DNA (CHEMICON International, Temecula, CA, USA) were used as controls for MSP. MSP products were separated electrophoretically on 3% agarose gels and stained with ethidium bromide. Signal intensities were measured with an image analyzer (model FIMBIO-2; Takara Bio, Otsu, Japan). We duplicated nested MSP in all samples revealing methylated DNA and randomly selected 50 samples not revealing methylated DNA in the first nested MSP. When methylated DNA was confirmed in both nested MSPs, we concluded that the gene was methylated in the sample.

Immunohistochemistry

DNMT1 protein expression level of each carcinoma was examined immunohistochemically in the ductal carcinomas of the same cohort in our previous study (21). The DNMT1 protein expression of each area microdissected from ductal carcinoma in the present study was re-evaluated as +, nuclear immunoreactivity for DNMT1 in 20% or ≥20% of microdissected carcinoma cells; and −, nuclear immunoreactivity for DNMT1 in <20% of microdissected carcinoma cells by reviewing previously stained formalin-fixed paraffin-embedded tissue slides.

Statistical analyses

Differences in the prevalence of DNA methylation of tumor-related genes among DE, DEI, PanIN and ductal carcinoma were analyzed by the χ2 test or Fisher direct analysis. Differences in the number of methylated genes among DE, DEI, PanIN and ductal carcinoma, and correlations between the number of methylated genes and tumor differentiation or DNMT1 protein expression, were analyzed by the Mann–Whitney U or Kruskal–Wallis tests. For all tests, P < 0.05 was considered to be the level of significance.

Results

DNA methylation status of each tumor-related gene

Figure 2 shows examples of the PCR products from MSP of each gene. Figure 3 shows the DNA methylation status of the 12 examined tumor-related genes in tissue samples from 10 representative cases of ductal carcinoma of the pancreas. If at least one of the multiple microdissected areas from an individual carcinoma showed DNA methylation of a particular gene, the carcinoma was considered to be positive for DNA methylation of that gene. DNA methylation profiles of the 58 ductal carcinomas are summarized in Figure 4. The prevalence of DNA methylation of each gene in DE, DEI, PanIN, and ductal carcinoma is summarized in Table 1 and Figure 5. No DNA methylation of the p14 gene was detected in any tissue samples analyzed. Although the BRCA1 and MGMT genes were methylated even in DE, methylation of other genes was never detected in DE tissue samples. In addition to the BRCA1 and MGMT genes, the p15, p16, APC, p73,
GSTP1 and TIMP-3 genes were also methylated in DEI and/or PanIN. All genes except the p14 gene were methylated in ductal carcinoma. Among the 12 genes examined, the BRCA1 (60.3%), APC (58.6%), p16 (39.3%) and TIMP-3 (30.9%) genes were the most frequently methylated in ductal carcinomas. The prevalence of DNA methylation of the APC and p16 genes increased progressively and significantly from DEI through PanIN to ductal carcinoma. On the other hand, the prevalence of DNA methylation of the BRCA1 gene was higher in DEI compared with ductal carcinoma, whereas DNA methylation of the TIMP-3 gene was prominent only in ductal carcinoma and never in DEI.

Stepwise accumulation of DNA methylation of tumor-related genes from DE through DEI and PanIN to ductal carcinoma

The prevalence of DNA methylation of at least one of the 12 genes and the average number of methylated genes were significantly higher in both DEI (60% and 0.85 ± 0.88, \( P = 0.0151 \) and \( P = 0.0224 \), respectively) and PanIN (67.5% and 0.95 ± 0.85, \( P = 0.0014 \) and \( P = 0.0028 \), respectively) than in DE (15.4% and 0.15 ± 0.38). The prevalence of DNA methylation of at least one of the 12 genes or the average number of methylated genes did not differ significantly between DEI and PanIN. There was no significant difference in either the prevalence of DNA methylation of at least one of the 12 genes or the average number of methylated genes among different grades of PanIN (PanIN IA, PanIN IB and PanIN II), probably owing to the small sample number of PanIN IA and II. The prevalence of DNA methylation of at least one of the 12 genes and the average number of methylated genes increased further from DEI and PanIN to ductal carcinoma (98.3% and 2.50 ± 1.35, \( P < 0.0001 \) and \( P < 0.0001 \) compared with both DEI and PanIN, respectively, Figure 6A and B). No significant correlation was observed between patient age and the prevalence of DNA methylation of at least one gene or the average number of methylated genes at any stage of carcinogenesis (data not shown). Stepwise accumulation of DNA methylation of tumor-related genes from DE through DEI and PanIN to ductal carcinoma was further confirmed in Figure 6C.

Heterogeneity of DNA methylation status in ductal carcinoma

DNA methylation profiles for 10 representative cases of the 23 ductal carcinomas from which 3–5 areas were microdissected are provided in Figure 3. From the 23 carcinomas, in total 19 areas of well-differentiated, 49 areas of moderately differentiated and 13 areas of poorly differentiated adenocarcinoma components were microdissected and analyzed. Of the 23 ductal carcinomas, 13, 15, 9, 9 and 9 carcinomas showed DNA methylation of the APC, BRCA1, p16, GSTP1 and TIMP-3 genes in at least one microdissected area, respectively. When all the 3–5 areas from an individual ductal carcinoma simultaneously showed DNA methylation of a particular gene, the carcinoma was regarded as negative for heterogeneity of the DNA methylation status of that gene (homoegenity). Otherwise, the carcinoma was regarded as positive for heterogeneity of the DNA methylation status of

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**Fig. 2.** Representative results of MSP of the 12 tumor-related genes in tissue samples. Positive bands under U and M (arrows) represent the unmethylated and methylated DNA of the corresponding genes in the left panel, respectively. PC, pancreatic carcinoma case; H2O, negative control for MSP using distilled water instead of template DNA; Posi, positive controls for MSP using universally unmethylated and methylated DNA; DE, DEI, peripheral pancreatic duct epithelia showing no remarkable histological changes without and with inflammatory background, respectively; TS1–TS5, multiple areas microdissected from the individual ductal carcinoma.
the gene. Of the 23 ductal carcinomas, heterogeneity of DNA methylation status was observed in 30.8% (4 out of 13) for the APC gene, 80% (12 out of 15) for the BRCA1 gene, 88.9% (8 out of 9) for the p16 gene, 100% (9 out of 9) for the GSTP1 gene and 66.7% (6 out of 9) for the TIMP-3 gene. The other less methylated genes also displayed considerable heterogeneity of DNA methylation status (data not shown). The average number of methylated genes per area increased significantly from well-differentiated (1.00 ± 0.88) through moderately differentiated (1.61 ± 1.02) to poorly differentiated (2.2 ± 1.13) cases.

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**Fig. 3.** DNA methylation status of the 12 tumor-related genes in tissue samples from 10 representative cases. DE, DEI, peripheral pancreatic duct epithelia showing no remarkable histological changes without and with inflammatory background, respectively; TS1–TS5, multiple areas microdissected from the individual ductal carcinoma; WD, MD and PD, well, moderately and poorly differentiated adenocarcinoma components, respectively; black grid squares, methylated sites; open grid squares, unmethylated sites; open grid squares with slash, failed or not done. DNMT1 protein expression was evaluated here by reviewing each exact microdissected area in the individual ductal carcinoma from our previously immunohistochemically stained tissue specimens (24). (+), nuclear immunoreactivity for DNMT1 in 20% or >20% of microdissected carcinoma cells; (−), nuclear immunoreactivity for DNMT1 in <20% of microdissected carcinoma cells.

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(2.31 ± 1.84) adenocarcinoma components in the 23 ductal carcinomas ($P = 0.0249$).

Significant correlation between accumulation of DNA methylation of multiple tumor-related genes and DNMT1 protein expression level in ductal carcinoma

DNMT1 protein expression level of each carcinoma was examined immunohistochemically in the ductal carcinomas of the same cohort in our previous study (21), and the results are shown in Figure 4. The average number of methylated genes per carcinoma was significantly higher in the 30 ductal carcinomas showing high-level DNMT1 expression, defined as nuclear immunoreactivity for DNMT1 in 20% or more of carcinoma cells all over the two to three representative slides (2.90 ± 2.09), than in the 28 ductal carcinomas showing low-level DNMT1 expression (2.07 ± 1.25, $P = 0.0324$, Figure 7A). To demonstrate this correlation more precisely, we re-evaluated DNMT1 nuclear immunoreactivity by reviewing the exact microdissected areas from previously stained tissue specimens of the 23 ductal carcinomas from which 3–5 areas were microdissected, and the results are shown in Figure 3. The significant correlation was confirmed for the microdissected areas: the average number of methylated genes per area was significantly higher in the 61 areas showing...
nuclear immunoreactivity for DNMT1 in 20% or more of microdissected carcinoma cells (1.77 ± 1.27) than in the 20 areas showing only low-level immunoreactivity (1.00 ± 0.795, \( P = 0.0093 \); Figure 7B).

Discussion

We believe that this is the first report of DNA methylation status in peripheral pancreatic duct epithelia, the exact origin of ductal carcinoma of the pancreas. It has been very difficult to examine molecular events in the originating tissue because it is a minute structure that can only be detected microscopically and is surrounded by abundant acinar cells. Because chronic pancreatitis is regarded as one of the risk factors for pancreatic carcinoma (13,14), at least part of peripheral pancreatic duct epithelia with inflammatory background may be at the precancerous stage. To examine DNA methylation status in tiny tissue samples of peripheral pancreatic duct epithelia without or with inflammatory background, avoiding contamination of surrounding acinar cells and/or lymphocytes, we

<table>
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The prevalence of DNA methylation increased progressively from DE, DEI, PanIN to ductal carcinoma \( (p < 0.01 \) and \( 2p < 0.05 \) by the \( \chi^2 \) test).

Fig. 5. The prevalence of DNA methylation of each of the 12 tumor-related genes in peripheral pancreatic duct epithelia showing no remarkable histological changes without (DE) and with (DEI) inflammatory background, PanIN and ductal carcinoma. The genes are classified into functional groups related to cell cycle control, DNA damage repair, tumor invasion, metastasis and apoptosis and are arranged following the order of decrease of the prevalence in ductal carcinoma from left to right in each group. \* \( P < 0.05 \), \* \* \( P < 0.01 \) by the \( \chi^2 \) test. For details, see Table I and the text.
employed a method combining tissue microdissection with agarose beads-based bisulfite conversion followed by nested MSP. This method also allowed us to examine DNA methylation status in PanIN, another precancerous lesion, and ductal carcinoma. Both the prevalence of DNA methylation and the average number of methylated genes were significantly increased from DE through DEI and PanIN to ductal carcinoma. Panel (C) shows the frequency distribution of DNA methylation of 0, 1 to 2 and 3 or >3 genes. Multiple gene (3 or >3 genes) methylation was observed more frequently in ductal carcinoma than in DEI or PanIN. Data are mean ± standard deviation.

Fig. 6. The prevalence of DNA methylation of at least one gene (A) and the average number of methylated genes (B) in peripheral pancreatic duct epithelia showing no remarkable histological changes without (DE) and with (DEI) inflammatory background, PanIN and ductal carcinoma. Both the prevalence of DNA methylation and the average number of methylated genes were significantly increased from DE through DEI and PanIN to ductal carcinoma. Panel (C) shows the frequency distribution of DNA methylation of 0, 1 to 2 and 3 or >3 genes. Multiple gene (3 or >3 genes) methylation was observed more frequently in ductal carcinoma than in DEI or PanIN. Data are mean ± standard deviation.

Fig. 7. Correlation between DNMT1 protein expression and the average number of methylated genes in ductal carcinomas of the pancreas. Significant correlation was observed not only in each carcinoma as a whole (A) but also in each microdissected area of carcinoma (B). Data are mean ± standard deviation.

carcinogenesis of the pancreas, even from the early precancerous stages, in both DEI and PanIN.

The APC and p16 genes were the most frequently methylated genes among the five cell cycle control genes examined, and the prevalence of DNA methylation of both genes increased progressively and significantly from DE through DEI and PanIN to ductal carcinoma, whereas they were never methylated in DE. The APC gene is known to be a very important component of the APC/β-catenin pathway (26). It has been reported that DNA methylation of the p16 gene actually results in silencing of the gene in various human tumors (27) as well as in PanIN (20) and pancreatic carcinomas (17). These data indicate that DNA methylation of the APC and p16 genes is particularly important during multistage carcinogenesis of the pancreas, even from the early precancerous stages.

A higher prevalence of DNA methylation of the BRCA1 gene was detected in DEI compared with PanIN and ductal carcinoma. BRCA1, a breast and ovarian cancer susceptibility gene, is involved in the maintenance of genome integrity by participating in processes such as homologous repair of double-stranded breaks (28). DNA methylation of the
promoter region of the BRCA1 gene has been reported in sporadic breast and ovarian carcinomas and was correlated with reduced mRNA and protein expression (29). Recently, Beger et al (30) reported reduced expression of the BRCA1 gene at both the mRNA and protein levels, not only in 50% of ductal carcinomas of the pancreas but also in pancreas with chronic pancreatitis. However, they did not provide a mechanism for this reduction of expression. The present findings suggest that DNA methylation may be the mechanism for downregulation of the BRCA1 gene, even in the pancreas with inflammatory background. The relatively high prevalence of DNA methylation of the BRCA1 gene in DEI suggests that inflammation may directly induce the efficient recruitment of DNA methylation machinery, such as DNA methyltransferases, to the promoter of the BRCA1 gene. In addition to methylation of the BRCA1 gene, DNA methylation of GSTP1, another so-called DNA caretaking gene that functions in the detoxification of carcinogens (31), was observed in DEI and was significantly increased in ductal carcinoma (Figure 5). Frequent and earlier DNA methylation of these DNA caretaking genes may make duct epithelia more susceptible to subsequent genetic and epigenetic changes promoting multistage carcinogenesis.

The TIMP-3 gene was frequently methylated in ductal carcinomas, whereas the prevalence of its methylation was rather low in PanIN and it was never methylated in DE and DEI, indicating that the TIMP-3 gene may play an important role in the invasion and metastasis of ductal carcinoma of the pancreas. The higher invasiveness of ductal carcinoma compared with intraductal papillary mucinous neoplasms (IPMN) of the pancreas is in accordance with the prevalence of DNA methylation of the TIMP-3 gene in ductal carcinoma (30.9% in the present study) and IPMN (0% in the study by House et al. (25)). Other differences in DNA methylation profiles between ductal carcinoma in the present study and IPMN in the study by House et al. (25) (such as higher prevalence of DNA methylation of the p73 and hMLH1 genes in IPMN, but higher prevalence of DNA methylation of the BRCA1 and APC genes in ductal carcinoma) are consistent with the different pathways of development and progression of these two types of carcinoma of the pancreas.

Low prevalence of DNA methylation of the BRCA1 and MGMT genes was detected even in DE. Because the DE samples used in this study were taken from patients with invasive ductal carcinoma of the pancreas, we cannot exclude the possibility that at least a part of the DE samples may have been exposed to the carcinogen causing ductal carcinoma. Otherwise, the BRCA1 and MGMT genes may be methylated in a tissue-specific and/or organ-specific manner in the pancreas.

Consistent with the considerable histological heterogeneity (32) and the previously reported heterogeneity of genetic alterations (33) in ductal carcinoma, considerable heterogeneity of the DNA methylation status of the 12 genes examined was observed among multiple microdissected areas from individual ductal carcinomas, suggesting that DNA methylation of tumor-related genes continues to accumulate in association with further subclonal malignant progression in an established ductal carcinoma. Notably, the number of methylated genes was significantly correlated with poorer tumor differentiation in each microdissected area from established ductal carcinomas. On the other hand, DNA methylation is an epigenetic event and, unlike gene mutation, can be reversible. This reversibility may also result in heterogeneity of DNA methylation status for a tumor-related gene after silencing of the gene has participated in an earlier stage of carcinogenesis. Among the 12 genes examined, the APC gene showed the lowest heterogeneity of DNA methylation status (30.8%), suggesting that DNA methylation of the APC gene may result in gene silencing that allows cells to obtain and retain growth advantages during the process of tumor clonal evolution.

We have reported that DNMT1 protein expression increased significantly and progressively from DE, DEI and PanIN to ductal carcinomas from this cohort used in our previous study (21). DNMT1 mRNA is expressed mainly during S-phase in normal cells and, because tumor tissue presumably contains a greater proportion of dividing cells than normal tissue, there has been some debate as to whether increased DNMT1 mRNA expression is due to an increase in the proportion of dividing cells or to an acute increase in DNMT1 expression per individual cell (34). However, DNMT1 protein overexpression was not associated with increased proliferative cell nuclear antigen (PCNA) labeling index in at least a proportion of DEI and PanIN and ductal carcinoma samples (data not shown). We have also previously observed a similar discrepancy between DNMT1 protein expression and cell proliferative activity in certain subgroups of gastric carcinomas (9). Moreover, DNMT1 protein expression has already been shown to be increased in urothelia at the precancerous stage of urinary bladder carcinoma, preceding any increase in the PCNA labeling index (10). Taken together, it appears that DNMT1 protein overexpression may not result entirely from an increase in the number of dividing cells during pancreatic carcinogenesis.

Moreover, progressive and significant accumulation of DNA methylation of tumor-related genes from DE through DEI and PanIN to ductal carcinoma is concordant with DNMT1 protein expression levels (21). Even in DEI, DNA methylation of some tumor-related genes and protein overexpression of DNMT1 occurred simultaneously. Accordingly, we detected a significant correlation between the average number of methylated genes and DNMT1 protein expression level in ductal carcinomas (Figure 7). DNMT1 targets replication foci, where DNA methylation patterns are copied from the mother strand, by binding to PCNA (35). However, targeting of the substrate DNA by DNMT1 may be disrupted by mechanisms such as dysfunction of p21WAF1 (36), which competes with DNMT1 for binding to PCNA, in carcinoma cells (35). In addition, it has recently been suggested that DNMT1 is capable of de novo methylation activity as well as having a maintenance function (37,38). In fact, DNMT1 protein overexpression has been reported to cause an increase of genomic 5-methylcytosine levels in human epithelial cells in vitro (39). Moreover, we have already reported that DNMT1 protein overexpression is associated with the CpG island methylator phenotype, defined by frequent DNA hypermethylation on C-type CpG islands (7), of stomach, colorectal and urinary bladder carcinomas (8,9,11). It is feasible that DNMT1 protein overexpression may be responsible for the accumulation of DNA methylation of tumor-related genes during multistage carcinogenesis of the pancreas, although the molecular mechanisms whereby overexpressed DNMT1 targets tumor-related genes require further clarification.

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References

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