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ORIGINAL ARTICLE

Dual epigenetic changes in diabetes mellitus-associated pancreatic ductal adenocarcinoma correlate with downregulation of E-cadherin and worsened prognosis

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Abstract

Diabetes mellitus (DM) is a risk factor for pancreatic ductal adenocarcinoma (PDAC) that promotes the promoter methylation of *CDH1*. It is still unclear whether DM can exert other epigenetic effects, such as altering microRNA (miR) expression, in PDAC. The expression of miR-100-5p is known to be changed in DM patients and can suppress the expression of E-cadherin. In this study, the correlation between DM status and dual epigenetic changes was evaluated in PDAC specimens from patients who underwent radical surgical resection. A total of 132 consecutive patients with PDAC were clinicopathologically evaluated. E-cadherin and nuclear β-catenin expression was measured using immunohistochemistry. DNA and miRs were extracted from the main tumor site on formalin-fixed paraffin-embedded tissue sections. TaqMan miR assays were applied to assess miR-100-5p expression. Bisulfite modification was conducted on the extracted DNA, which was then subjected to methylation-specific polymerase chain reaction. Immunohistochemistry revealed that decreased E-cadherin expression and increased nuclear β-catenin expression were significantly associated with DM and poor tumor cell differentiation. The presence of long-duration DM (≥3 years) was a significant factor contributing to *CDH1* promoter methylation ($p < 0.01$), while miR-100-5p expression was proportionally correlated with the preoperative HbA1c level ($R = 0.34$, $p < 0.01$), but not the duration of DM. The subjects with high miR-100-5p expression and *CDH1* promoter methylation showed the highest level of vessel invasion and prevalence of tumor size ≥30 mm. PDAC subjects with dual epigenetic changes showed poorer overall survival (OS) than those with a single epigenetic change. miR-100-5p expression ≥4.13 and *CDH1* promoter methylation independently predicted poor OS and disease-free survival (DFS) in the multivariate analysis. OS and DFS worsened in DM subjects with both HbA1c ≥ 6.5% and DM duration ≥3 years. Thus, DM is associated with two modes of epigenetic change by independent mechanisms and worsens prognosis.

Keywords: diabetes mellitus; pancreatic ductal adenocarcinoma; promoter methylation; microRNA; E-cadherin

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains an intractable cancer with a poor prognosis. The 5-year survival rate of PDAC is low at approximately 10% [1]. Systemic treatment in addition to local control is clearly essential to curing PDAC, while surgical resection of PDAC is still a predominant treatment option. Genetic studies have identified mutations of four key genes, including *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*, as responsible for the development of PDAC [2–6]. However, targeting these genetic variations has yet to produce a useful therapeutic strategy to combat PDAC. Epigenetic changes are also involved in the tumorigenesis of PDAC [7]. Interventions related to epigenetic changes are expected to lead to the development of new therapeutic strategies for PDAC.

Diabetes mellitus (DM) has long been known to be a risk factor for the development of PDAC [8-10]. DM elicits epigenetic changes, such as promoter methylation or microRNA (miR) expression changes, related to epithelial–mesenchymal transition (EMT) [11-14]. Loss of E-cadherin leads to nuclear translocation of β-catenin, poor prognosis, and the development of resistance to chemotherapy in PDAC patients via induction of EMT [15-18]. Regarding PDAC associated with DM, our previous studies reported that a long duration of DM (long-DM; 3 years and more) exacerbates the frequency of *CDH1* promoter methylation, stellate cell activation, and EMT [10,19], which worsens the prognosis and enhances the malignant behavior of PDAC. Nevertheless, it has yet to be clarified how DM is implicated in the comprehensive epigenetic changes, including miR expression and promoter methylation of *CDH1* in PDAC.

miRs are small, noncoding endogenous RNAs that mainly function as negative regulators of gene expression. miR-100, also referred to as miR-100-5p, is upregulated in PDAC tissue and promotes the EMT of PDAC cells, resulting in disease progression [20–24]. Moreover, in diabetic subjects, the expression of miR-100 in blood exosomes of pre-type 2 DM subjects was increased compared to that in those of nondiabetic subjects [25], while the expression in T cells and serum of type 1 DM subjects and visceral fatty tissue in type 2 DM was decreased [26–28]. Therefore, it is still unknown how miR-100 is involved in the progression of PDAC associated with DM.

Herein, we focused on the involvement and association of epigenetic changes in E-cadherin, including promoter methylation and miR-100-5p expression in PDAC complicated with DM. Our study also provides novel therapeutic options for PDAC complicated with DM.

Materials and methods

Case selection

A total of 132 consecutive subjects who underwent surgical resection for PDAC were evaluated between 2014 and 2019 in Hirosaki University Hospital, and patients with unresectable disease and distant metastasis at diagnosis were excluded. Clinicopathological information was obtained from the medical records. The HbA1c value at a single time point (within 1 month before surgery) was used for clinicopathological evaluation, because it was proportionally correlated with average HbA1c values at multiple points (3–12 months before surgery; supplementary material, Figure S1). DM was confirmed from the medical records and was diagnosed based on the criteria proposed by the Japan Diabetes Society [29]. According to our previous reports, long-DM was defined as DM lasting 3 years or more, and DM lasting less than 3 years was defined as short-duration DM (short-DM) [10,19]. This study was approved by the ethical committee of Hirosaki University Graduate School of Medicine (approval number: 2020-143).

Histopathological assessment

Screening of pathological findings was performed with H&E sections in each subject. The pathological diagnosis of PDAC was re-evaluated according to the 2019 WHO classification of tumors of the digestive system and graded based on the Union for International Cancer Control tumor-node-metastasis classification of malignant tumors (eighth edition) by two pathologists (HM and KK) [30]. Histologic grade was divided into three categories of well-differentiated carcinoma (well), moderately differentiated adenocarcinoma (mod), and poorly differentiated adenocarcinoma (poor) based on the degrees of tubular formation, mucin production, and mitoses. The highest grade in the sections represented the histological grade of the individuals regardless of the proportion. Venous invasion was assessed on tumor sections stained with Elastica-van Gieson. Lymphatic invasion was evaluated on immunostained sections for podoplanin (clone D2-40, 1:4, Nichirei Bioscience Inc., Tokyo, Japan). The degree of invasion into venules and lymph vessels was graded as 0 (none), 1 (1–3 sites), 2 (4–6 sites), and 3 (>6 sites) within 10 high-power fields.

Immunohistochemical analysis

For immunohistochemistry, the standard streptavidin–biotin technique was applied to the sections using the Benchmark Ultra Automated Slide Preparation system (Ventana Medical Systems, Inc., Tucson, AZ, USA). Antibodies against E-cadherin (clone NCH-38, 1:100 dilution, Agilent Technologies, Santa Clara, CA, USA) and β-catenin (clone 17C2, 1:100 dilution, Leica Biosystems, Deer Park, IL, USA) were used. Negative control stains were performed by omitting the primary antibodies or substituting nonimmune rabbit or swine sera. Normal pancreatic tissue in each section was used as a positive control. E-cadherin expression patterns were classified into three categories according to the definition by Saito *et al*: loss of expression, <5% of the tumor cells were stained;

reduced expression, 5–49% of the tumor cells were stained; and preserved expression, 50% or more tumor cells were stained [10]. Cases showing reduced or lost E-cadherin expression were defined as 'low E-cadherin expression' cases. For β-catenin expression, a total of 300 cancer cells were counted in each section. The number of carcinoma cells showing nuclear staining for β-catenin was assessed in relation to the total number of carcinoma cells and expressed as a percentage regardless of the staining intensity (referred to as the β-catenin index) [31].

Evaluation of *CDH1* promoter methylation by methylation-specific polymerase chain reaction

Methylation-specific PCR (MS-PCR) was carried out according to a previous protocol [10,32]. Tissue samples included in this study had >60% viable cells and <20% necrosis. Tumor areas without hemorrhage or severe inflammation and areas adjacent to the tumor (6 cm^2 on average) were selected for MS-PCR evaluation. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections (10 μm) following the protocol of the DNA extraction kit for FFPE (Qiagen K.K., Tokyo, Japan). Subsequently, bisulfite modification was conducted on the extracted samples with a commercially available kit (EpiTect Fast Bisulfite Conversion Kits, Qiagen K.K.), and they were then subjected to MS-PCR with identical primer sequences to Herman *et al* using a Taq DNA polymerase designed for bisulfite PCR (EpiTaq™ HS, TAKARA BIO INC., Shiga, Japan) [33]. The amplicons were analyzed by electrophoresis on a 3% agarose gel. A positive methylated band indicated high rates of methylation of the CpG region.

Analysis of miR-100-5p expression by real-time PCR

FFPE tumor tissue samples with 40–60% cancer cells were eligible for miR analysis. Total RNA enriched in the miR fraction was purified by using the miRNeasy FFPE kit isolation system following the manufacturer's protocols (Qiagen K.K.). The RNA samples with OD260/OD280 ratios ranging between 1.8 and 2.0 were considered to be of good quality. Reverse transcription and quantitative PCR for miR-100-5p and the endogenous control RNU6B were conducted by using TaqMan MicroRNA Assays (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR measurements with specific TaqMan primers for each miR were performed in duplicate on the ABI PRISM 7000 system (Applied Biosystems, Foster City, CA, USA), determining a mean Ct value for each sample. The relative expression of miR-100-5p was calculated using the comparative Ct method [34,35].

Statistical analysis

All statistical analyses were conducted using the free statistics application EZR (ver. 1.55, https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html). Disease-free survival (DFS) was defined as the time elapsed between surgical resection and tumor recurrence. Overall survival (OS) was calculated as the time between surgery and death from all causes. Continuous variables were compared with Student's t test or the Mann–Whitney U test. Categorical variables were compared by chi-square analysis or the Mann–Whitney U test, where appropriate. Comparisons of average values between two groups were analyzed by the non-parametric Mann–Whitney U test. For multiple comparisons, the Z test with Bonferroni adjustment was used. Survival curves were calculated with Kaplan–Meier analysis, and p values were determined by the log-rank test for censored survival data. Multivariate survival analysis was performed by the Cox proportional hazard model. All tests were two-tailed, and a p value of <0.05 was considered statistically significant.

Results

Clinical and pathological characteristics

There were no significant differences in sex, age, smoker proportion, tumor size, T stage, N stage, histologic grade, lymph vessel and venous invasion (ly and v factors), nerve invasion (ne factor), INF type, curative resection, neoadjuvant chemotherapy ratio, postadjuvant chemotherapy ratio, resectability or CA19-9 level between the control and DM groups (supplementary material, Table S1). Body mass index (BMI) was higher in DM patients than in non-DM patients (23.5 versus 21.8, $p < 0.01$). In terms of the location of the tumor, the pancreatic head was more common in control patients (75%) than in DM patients (54%; $p < 0.01$). Pancreatico-duodenectomy was performed more often in control patients (72%) than in DM patients (51%; $p < 0.05$).

E-cadherin protein expression and nuclear translocation of β-catenin in PDAC were decreased in patients who also had DM

In H&E sections of nonneoplastic areas in each group, ducts were composed of typical monolayered epithelial

cells (Figure 1A). In the adjacent tumor area, the invasive growth of carcinoma cells with irregular ductal structures mixed with dense fibrous stroma was observed in all PDAC lesions (Figure 1B). Carcinoma cells exhibiting diffuse or solid growth were diagnosed as poorly differentiated carcinoma (Figure 1C). Immunohistochemical analysis revealed that E-cadherin was expressed on the cell membrane of nonneoplastic ductal cells, acinar cells, and tumor cells (Figure 1D,E). E-cadherin expression was low around the cell membrane in poorly differentiated PDAC samples (Figure 1F). β-Catenin was localized in the cell membrane of non-DM and short-DM subjects, while nuclear and cytosolic expression of β-catenin was more evident in long-DM subjects (Figure 1G–I). Semiquantitative evaluation of cancerous tissues revealed that the prevalence of low E-cadherin expression was significantly increased in poorly differentiated PDAC (versus moderately differentiated to well-differentiated PDAC; $p < 0.05$; Figure 1J). Low E-cadherin was significantly more common in the DM group than in the non-DM group (57% versus 33%, $p < 0.01$; Figure 1K). Low E-cadherin expression was significantly more common in subjects with high levels of HbA1c (≥6.5%) and long-DM ($p < 0.05$; Figure 1L,M). The β-catenin index was significantly increased in poorly differentiated PDAC (versus moderately differentiated to well differentiated PDAC; $p < 0.01$; Figure 1N), the DM group (versus the non-DM group; $p < 0.01$; Figure 1O), and patients with high levels of HbA1c and long-DM ($p < 0.01$; Figure 1P,Q).

The frequency of *CDH1* promoter methylation was increased in patients with long-DM

The frequency of *CDH1* promoter methylation in PDAC evaluated by MS-PCR was significantly increased in the DM group compared to the control group (56.9% versus 43.1%, $p < 0.05$; Table 1). When the DM groups were compared with the remaining subjects, no increase in the frequency was observed in those with short-DM (43.8% versus 56.3%, $p = 0.69$), while the frequency was significantly increased in PDAC tissues in those with long-DM (69.7% versus 30.3%, $p < 0.01$). In contrast, HbA1c level had no impact on the prevalence of *CDH1* promoter methylation. DM treatment [diet therapy, metformin, dipeptidyl peptidase 4 (DPP4) inhibitor, and insulin] did not influence the prevalence. The prevalence of *CDH1* promoter methylation was significantly increased in the E-cadherin low group (39.0% versus 61.0%, $p < 0.01$).

The proportion of patients with long-DM was significantly higher among the subjects with *CDH1* promoter methylation than among those without *CDH1* promoter methylation ($p < 0.05$; Figure 2A). The DM subjects with *CDH1* promoter methylation showed a significantly higher β-catenin index than those without DM and DM subjects without *CDH1* promoter methylation ($p < 0.01$ and $p < 0.05$, respectively; Figure 2B). The HbA1c level in DM subjects was significantly higher than that in non-DM subjects regardless of the presence of *CDH1* promoter methylation ($p < 0.01$, respectively; Figure 2C).

miR-100-5p expression was significantly correlated with preoperative HbA1c levels

miR-100-5p expression was higher in PDAC subjects with DM than in those without DM (expression value: 3.5 versus 6.0, $p < 0.001$; Table 2). In contrast to the effect on *CDH1* promoter methylation, long-DM had a marginal impact on miR-100-5p expression (6.0 versus 3.7, $p = 0.06$); rather, short-DM significantly increased miR-100-5p expression (5.9 versus 4.0, $p < 0.001$). miR-100-5p expression was higher in the high HbA1c group than in the low HbA1c group (6.0 versus 3.7, $p < 0.001$). The subjects treated with the DPP4 inhibitor showed increased expression compared to nontreated subjects (6.0 versus 3.9, $p < 0.01$). The subjects exhibiting low expression of E-cadherin showed higher expression of miR-100-5p than those with high E-cadherin expression (6.0 versus 3.9, $p < 0.001$). miR-100-5p expression was not influenced by *CDH1* promoter methylation (5.2 versus 3.9, $p = 0.17$).

In the bivariate correlation analysis, there was no correlation between DM duration and miR-100-5p expression ($r = 0.06$, $p = 0.70$; Figure 2D). The expression level of miR-100-5p was proportionally correlated with the β-catenin index ($r = 0.35$, $p < 0.001$; Figure 2E). The expression level of miR-100-5p was significantly correlated with preoperative HbA1c value ($r = 0.34$, $p < 0.001$; Figure 2F).

Clinicopathological features associated with multiple epigenetic changes in subjects with PDAC associated with DM

The proportion of subjects with high CA19-9 (≥200 U/ml) was significantly higher in the groups showing a single epigenetic change (S-epi) and dual epigenetic changes (D-epi) than in the group with no epigenetic changes (N-epi; $p < 0.01$ versus N-epi, respectively; Table 3). The proportion of males was

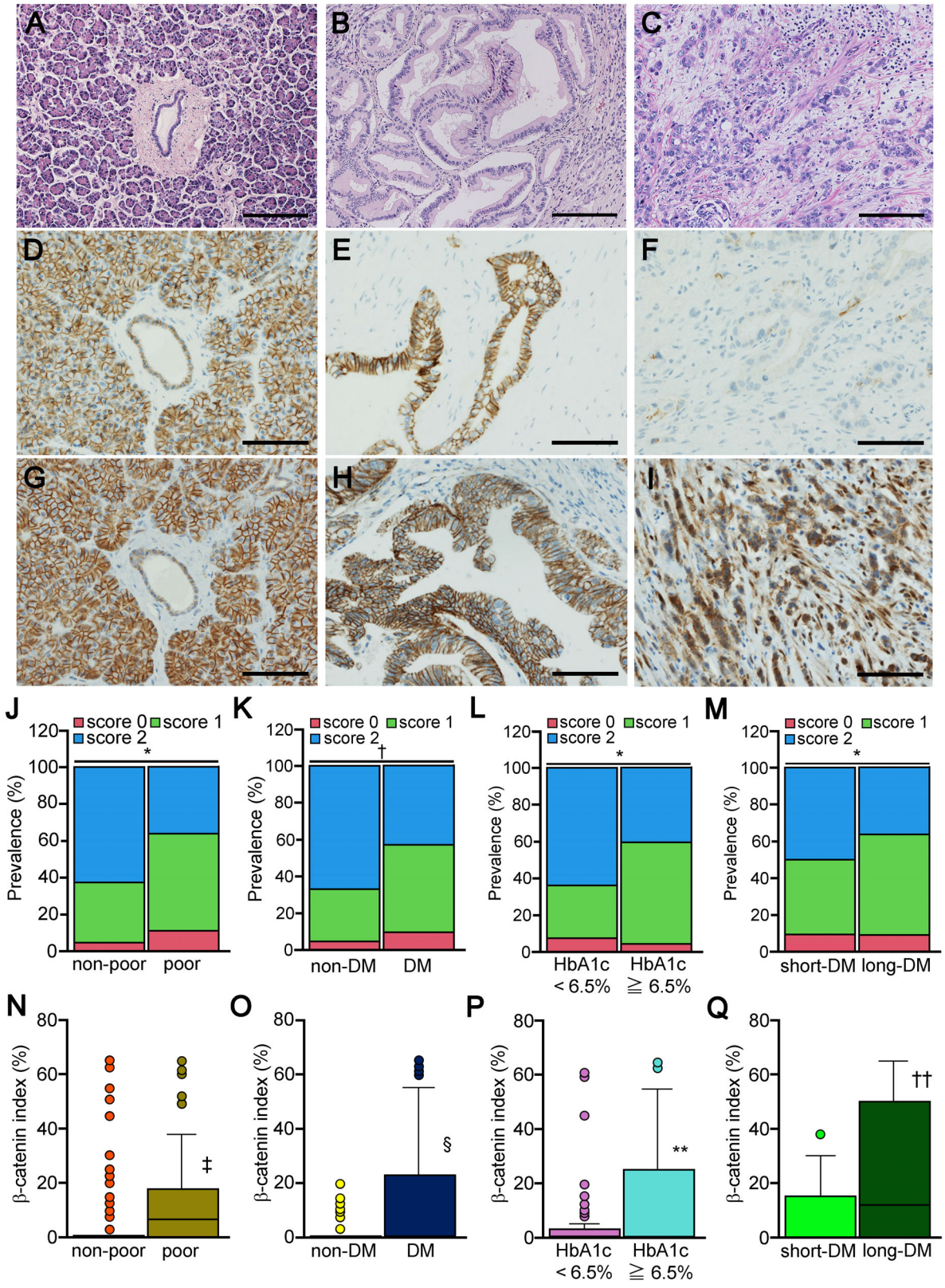


Figure 1. Legend on next page.

Table 1. *CDH1* promoter methylation analysis.

	Methylation− (cases)	Methylation+ (cases)	*P* value
Age ≧ 65 years	57.7% (60/104)	42.3% (44/104)	0.054
Male	51.6% (33/64)	48.4% (31/64)	
Female	54.4% (37/68)	45.6% (31/68)	0.862
BMI ≧ 25 kg/m^2	48.6% (17/35)	51.4% (18/35)	0.559
DM	43.1% (28/65)	56.9% (37/65)	<0.05
Short-DM	56.3% (18/32)	43.8% (14/32)	0.690
Long-DM	30.3% (10/33)	69.7% (23/33)	<0.01
HbA1c ≧ 6.5%	42.2% (19/45)	57.8% (26/45)	0.098
Diet therapy	45.5% (10/22)	54.5% (12/22)	0.488
Metformin user	37.5% (3/8)	62.5% (5/8)	0.474
DPP4 inhibitor user	41.2% (14/34)	58.8% (20/34)	0.116
Insulin user	36.4% (4/11)	63.6% (7/11)	0.347
Smoker	46.4% (32/69)	53.6% (37/69)	0.120
E-cadherin, low	39.0% (23/59)	61.0% (36/59)	<0.01

significantly higher in the D-epi group than in the N-epi group ($p < 0.05$). The prevalence of poorly differentiated carcinoma (poor), ly2-3, and low E-cadherin expression gradually increased along with the number of epigenetic changes in the subjects with PDAC complicated with DM. The prevalence of tumor size ≥30 mm and N1, borderline resectable, v2-3, and ne2-3 tumors was significantly higher in the D-epi group than in the N-epi and S-epi groups.

Epigenetic status was associated with short OS and DFS in PDAC

Univariate analysis of OS showed that tumor size ≥30 mm ($p < 0.05$), N1 ($p < 0.05$), CA19-9 ≥ 200 U/ml ($p < 0.01$), adjuvant chemotherapy ($p < 0.01$), HbA1c ≥ 6.5% ($p < 0.05$), long-DM ($p < 0.05$), miR-100-5p ≧ 4.13 ($p < 0.05$), and *CDH1* promoter methylation ($p < 0.01$) were significant risk factors for shorter survival (Table 4). Tumor size ≥30 mm ($p < 0.05$), adjuvant chemotherapy ($p < 0.01$), miR-100-5p ≧ 4.13, and *CDH1* promoter methylation ($p < 0.05$) were independent predictors of OS in the multivariate analysis (Table 5). Univariate analysis of DFS showed that tumor size ≥30 mm ($p < 0.05$), N1 ($p < 0.001$), CA19-9 ≥ 200 U/ml ($p < 0.01$), adjuvant chemotherapy ($p < 0.001$), HbA1c ≥ 6.5% ($p < 0.05$), miR-100-5p ≥ 4.13 ($p < 0.05$), and *CDH1* promoter methylation ($p < 0.01$) were significant risk factors for shorter survival (supplementary material, Table S2). Tumor size ≥30 mm ($p < 0.05$), CA19-9 ≥ 200 U/ml ($p < 0.05$), adjuvant chemotherapy ($p < 0.01$), miR-100-5p ≧ 4.13 and *CDH1* promoter methylation ($p < 0.05$) were independent factors for DFS in multivariate analysis (supplementary material, Table S3). The Kaplan–Meier survival curves clearly indicated that prognosis in terms of OS worsened as the number of epigenetic modifications increased (Figure 3A). The number of epigenetic modifications was indirectly associated with worsened DFS ($p < 0.05$, N-epi versus S-epi and $p < 0.01$ N-epi versus D-epi; Figure 3B). OS and DFS in the subjects with both high-HbA1c and long-DM were significantly worse than those in the remaining diabetic subjects (others; $p < 0.01$ and $p < 0.05$, respectively; Figure 3C,D).

Discussion

In this study, we first found that multiple epigenetic processes were associated with the development of PDAC associated with DM. *CDH1* promoter methylation was associated with the long-DM, while the prevalence did not correlate with HbA1c level. Moreover, the expression of miR-100-5p correlated positively

Figure 1. Histology of pancreatic ductal adenocarcinoma and E-cadherin expression. (A) In H&E sections, the epithelial cells in the duct showed no atypia and monolayered arrays in the nonneoplastic area. (B) PDAC cells exhibited an irregular ductal pattern with stromal reaction and were diagnosed as well or moderately differentiated adenocarcinoma. (C) PDAC cells exhibiting diffuse or solid growth were diagnosed as poorly differentiated carcinoma. (D) Immunohistochemical analysis revealed that E-cadherin was strongly expressed on the cell membrane of nonneoplastic ductal cells. (E) Moderately differentiated PDAC cells showed robust E-cadherin expression on the cell membrane, while (F) there was no E-cadherin expression in poorly differentiated PDAC cells, judged as E-cadherin loss. β-Catenin was robustly expressed on the cell membrane of (G) nonneoplastic ductal cells and (H) moderately differentiated PDAC cells, while nuclear expression of β-catenin was evident in (I) poorly differentiated PDAC cells. Semiquantitative evaluation of cancerous tissues revealed that (J) low E-cadherin expression was significantly increased in poorly differentiated compared to non-poorly differentiated tumors. The prevalence of low E-cadherin subjects was significantly higher (K) in the DM group than in the non-DM group, (L) the HbA1c ≥ 6.5% group than in the HbA1c < 6.5% group, and (M) the long-DM group than in the short-DM group. The β-catenin index was significantly higher in (N) poorly differentiated tumors compared with non-poorly differentiated tumors, the (O) the DM group versus the non-DM group, (P) the HbA1c ≥ 6.5% group versus the HbA1c < 6.5% group, and (Q) the long-DM group versus the short-DM group. Significance: $^{*}p < 0.05$, $^{\dagger}p < 0.01$ (chi-square test), $^{\ddagger}p < 0.01$ versus non-poor, $^{\S}p < 0.01$ versus non-DM, $^{**}p < 0.01$ versus HbA1c < 6.5%, and $^{\dagger\dagger}p < 0.01$ versus long-DM. Scale bars are 200 μm (A–C) and 100 μm (D–I).

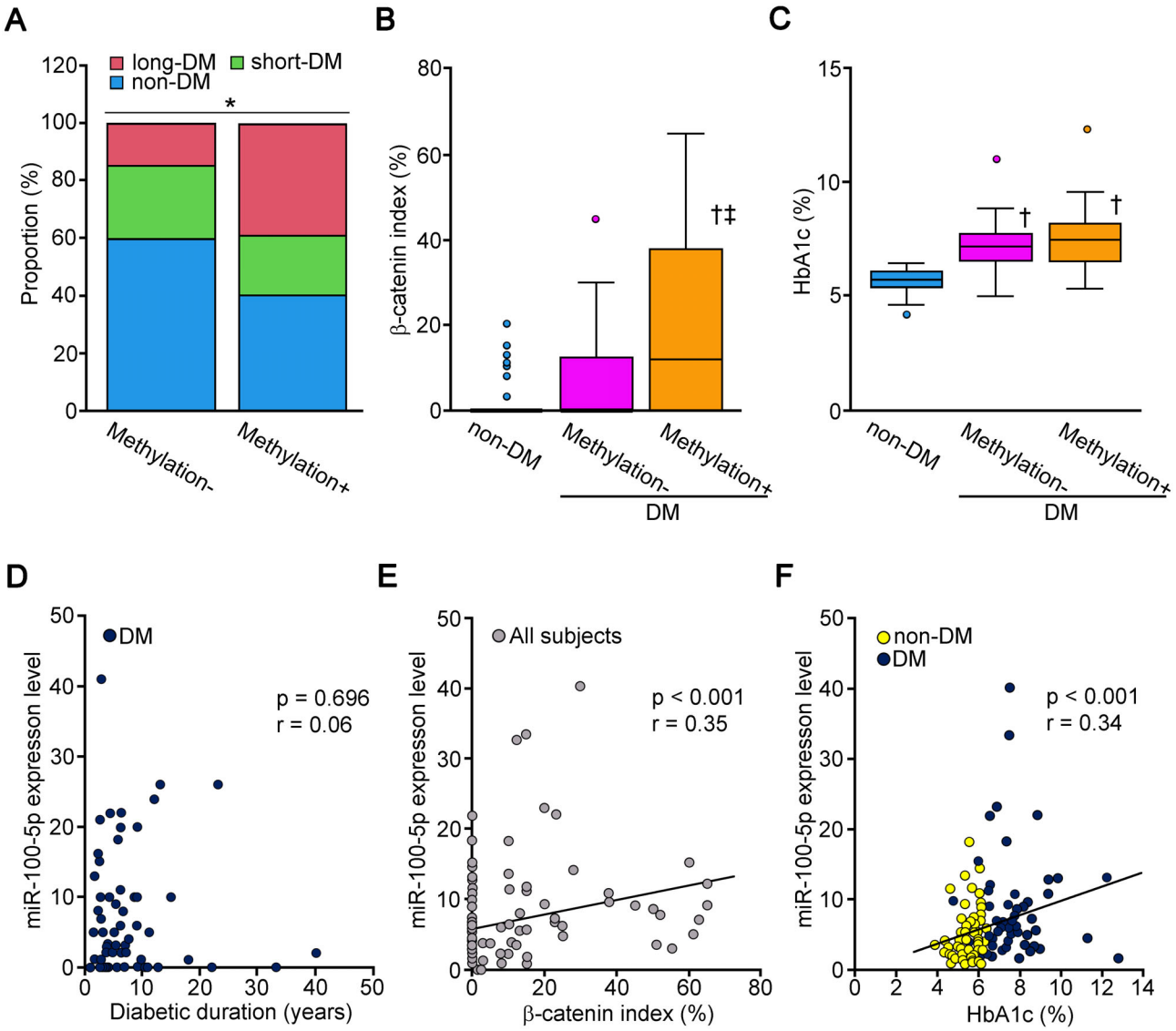


Figure 2. Correlation between epigenetic changes and diabetes-related measurements. (A) The prevalence of a long duration of DM (≥3 years) was significantly higher in PDAC subjects with *CDH1* promoter methylation than in PDAC subjects without *CDH1* promoter methylation. (B) The β-catenin index was significantly higher in DM subjects with *CDH1* promoter methylation than in non-DM subjects and DM subjects without *CDH1* promoter methylation. (C) The HbA1c level was significantly higher in DM subjects than in non-DM subjects regardless of the presence of *CDH1* promoter methylation. The HbA1c level was comparable between the subjects with *CDH1* promoter methylation − and +. (D) The duration of DM did not correlate significantly with the expression level of miR-100-5p ($r = 0.06$, $p = 0.70$). (E) The expression level of miR-100-5p was proportionally correlated with the β-catenin index in all subjects ($r = 0.35$, $p < 0.001$). (F) The expression level of miR-100-5p was proportionally correlated with the HbA1c level in subjects with PDAC complicated with DM ($r = 0.34$, $p < 0.001$). Significance: $^{*}p < 0.05$ (chi-square test), $^{\dagger}p < 0.01$ versus non-DM, $^{\ddagger}p < 0.05$ versus DM methylation−.

with preoperative HbA1c but not with the duration of DM. Both variables were strongly correlated with suppressed expression of E-cadherin in PDAC subjects. The decrease in E-cadherin expression was accompanied by an increase in the β-catenin index. PDAC subjects with double positivity for *CDH1* promoter methylation and high expression of miR-100-5p showed a significantly greater prevalence of tumor size ≥30 mm and N1, borderline resectable, poorly differentiated, v2-3, ly2-3, ne2-3, and E-cadherin expression-low

Table 2. miR-100-5p analysis.

		No.	miR-100-5p median (25–75% IQR)	*P* value
Age (year)	<65	29	3.8 (3.1–7.0)	
	≧65	103	4.3 (2.9–7.6)	0.724
Sex	Male	65	3.9 (2.9–6.0)	
	Female	67	5.1 (3.0–9.0)	0.124
BMI (kg/m^2)	<25	97	4.2 (2.9–7.4)	
	≧25	35	4.1 (3.1–6.6)	0.920
DM	−	67	3.5 (2.4–5.4)	
	+	65	6.0 (3.6–9.2)	<0.001
Short-DM	−	99	4.0 (2.7–6.7)	
	+	33	5.9 (3.8–10.8)	<0.001
Long-DM	−	98	3.7 (2.7–9.0)	
	+	34	6.0 (3.3–9.0)	0.061
HbA1c (%)	<6.5	81	3.7 (2.7–6.3)	
	≧6.5	51	6.0 (4.1–9.4)	<0.001
Diet therapy	−	22	4.1 (2.9–6.9)	
	+	110	6.0 (3.8–10.5)	0.089
Metformin user	−	124	4.1 (2.9–7.3)	
	+	8	6.5 (4.7–9.1)	0.198
DPP4 inhibitor user	−	97	3.9 (2.7–6.9)	
	+	35	6.0 (3.7–8.9)	<0.01
Insulin user	−	119	4.1 (2.9–7.4)	
	+	13	6.0 (3.5–7.1)	0.450
Smoker	−	63	3.8 (2.5–7.1)	
	+	69	4.3 (3.2–7.6)	0.469
E-cadherin expression	High	83	3.9 (2.7–5.5)	
	Low	59	6.0 (2.7–9.2)	<0.001
CDH1 promoter methylation	−	68	3.9 (2.7–6.4)	
	+	64	5.2 (3.2–8.3)	0.174

IQR, interquartile range.

Table 3. Clinicopathological characteristics related to epigenetic changes.

Degree of epigenetic change	N-epi	S-epi	D-epi
Age ≧ 65 years	69.2% (9/13)	82.8% (24/29)*	80.0% (20/25)
Male	30.8% (4/13)	44.8% (13/29)	48.0% (12/25)*
Female	69.2% (9/13)	55.2% (16/29)	52.0% (13/25)
CA19-9 ≧ 200 U/ml	15.4% (2/13)	35.5% (10/29)†	36.0% (9/25)†
Tumor size ≧ 30 mm	38.5% (5/13)	37.9% (11/29)	60.0% (15/25)†,‡
N1	46.2% (6/13)	51.7% (15/29)	68.0% (17/25)†,§
Borderline resectable	15.4% (2/13)	10.3% (3/29)	32.0% (8/25)†,‡
Poorly differentiated	7.7% (1/13)	20.7% (6/29)*	52.0% (13/25)†,‡
v2-3	69.2% (9/13)	72.4% (21/29)	96.0% (24/25)†,‡
ly2-3	46.2% (6/13)	62.1% (18/29)*	80.0% (20/25)†,‡
ne2-3	84.6% (11/13)	89.7% (26/29)	100% (25/25)†,‡
E-cadherin expression, low	30.8% (4/13)	55.2% (16/29)†	72.0% (18/25)†,§

D-epi, subjects showing dual epigenetic change; N-epi, subjects showing non-epigenetic change; S-epi, subjects showing single epigenetic change.
*$P < 0.05$ versus N-epi.
†$P < 0.01$ versus N-epi.
‡$P < 0.01$ versus S-epi.
§$P < 0.05$ versus S-epi.

tumors than subjects with negative or single-positive tumors. The OS of double-positive subjects was significantly worse than that of negative or single-positive subjects. The subjects with a long-DM and high HbA1c showed significantly worse OS and DFS than the remaining subjects with PDAC associated with DM.

miR-100 expression differs depending on DM state and tissue type [25–28]. This suggests that miR-100-5p expression widely varies in different diabetic states and that E-cadherin expression can be altered in patients with a relatively short duration of DM depending on HbA1c levels. E-cadherin expression mediated by miR-100-5p may be reversible and preventable with glycemic control in PDAC patients with DM.

On the other hand, as shown in our previous study, a long-DM was associated with a high prevalence of *CDH1* promoter methylation but not high HbA1c levels in PDAC subjects. Although the precise mechanism is still speculative, prolonged metabolic disorder can cause epigenetic alterations in diabetic patients [36]. It is generally known that the prevalence of diabetic complications can increase along with diabetic progression [37]. The pathophysiology of diabetic complications is chronic inflammation, the production of advanced glycation end products and activation of their signaling, increased levels of oxidative stress, and tissue

Table 4. Univariate analysis (OS).

Factor	Median OS (date)	*P* value
Age (years): <65 versus ≧65	943 versus 979	0.528
Male versus female	711 versus 1,166	0.111
Location: body-tail versus head	1,166 versus 943	0.52
Tumor size (mm): <30 versus ≧30	1,010 versus 711	<0.05
T1–2 versus T3–4	979 versus 847	0.216
N: N0 versus N1	1,184 versus 646	<0.05
CA19-9 (U/ml): <200 versus ≧200	1,097 versus 602	<0.01
Adjuvant chemotherapy: (−) versus (+)	537 versus 1,163	<0.01
HbA1c (%): <6.5 versus ≧6.5	1,166 versus 742	<0.05
DM: (−) versus (+)	1,184 versus 847	0.074
Long-DM: (−) versus (+)	1,066 versus 632	<0.05
miR-100-5p expression: <4.13 versus ≧4.13	1,184 versus 703	<0.05
CDH1 promoter methylation: (−) versus (+)	1,001 versus 602	<0.01

Table 5. Multivariate analysis (OS).

Factor	Risk ratio	95% CI	*P* value
Tumor size (mm) ≧30	1.73	1.06–2.81	<0.05
N: N0 versus N1	1.20	0.72–2.00	0.48
CA19-9 (U/ml) ≧200	1.43	0.87–2.34	0.17
Adjuvant chemotherapy (+)	0.37	0.21–0.65	<0.01
miR-100-5p expression ≧4.13	1.80	1.12–2.92	<0.05
CDH1 promoter methylation (+)	1.64	1.02–2.66	<0.05

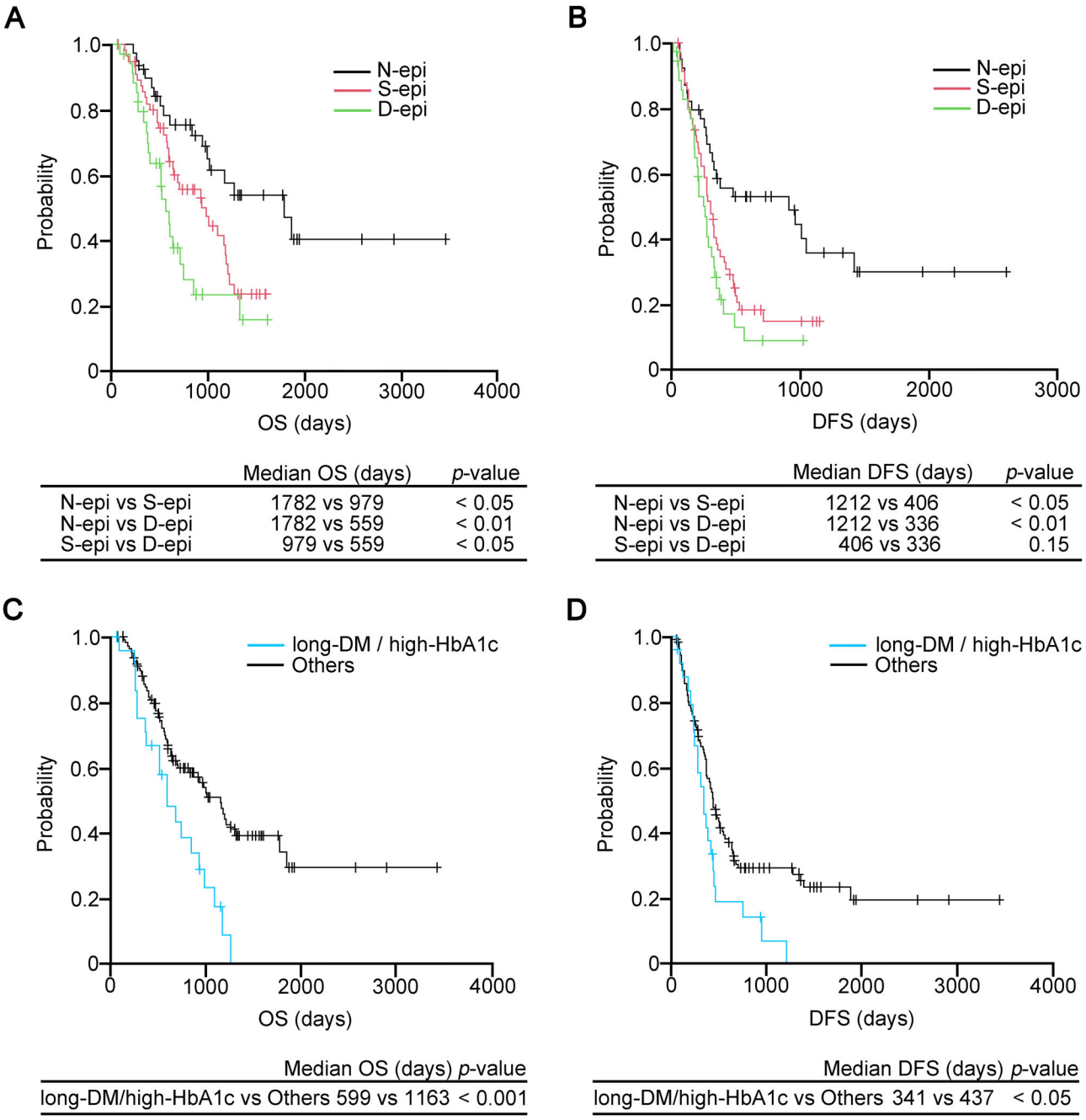
ischemia exacerbated by micro- and macroangiopathy, which include some of the triggers of promoter methylation [38–42]. Furthermore, the duration of diabetes changes the intratumoral composition of inflammatory cells in PDAC, which may increase the level of *CDH1* promoter methylation [43]. Thus, additional insults evoked by long-DM may be involved in promoter methylation in PDAC. These findings may suggest the possibility that the regulation of *CDH1* promoter methylation in PDAC patients with long-DM is more difficult than repression of miR-100-5p expression, which varies only with HbA1c levels.

E-cadherin downregulation has been described as a major requirement for aggressive malignant behavior such as invasion eliciting EMT [44]. Interestingly, dual epigenetic changes in E-cadherin significantly worsened OS compared to a single epigenetic change in PDAC. This suggests that the number of epigenetic changes is not highly involved in PDAC tumorigenesis itself; rather, these changes may increase tumor aggressiveness and subsequent chemoresistance. It is known that the degree of methyl base addition in CpG islands of *CDH1* promoter can vary between cases [45]. Therefore, high expression of miR-100-5p would complementarily suppress E-cadherin expression and induce robust EMT, leading to poor OS in PDAC cell patients with dual epigenetic changes.

Surgery is the only potentially curative treatment for PDAC. However, due to its early metastatic nature, only up to 20% of patients are candidates for initial resection of PDAC [46]. Therefore, there is an urgent need to develop new methods for PDAC diagnosis and recurrence monitoring. Liquid biopsy is a noninvasive cancer detection method involving the detection of microvesicles and exosomes containing nucleic acids released into body fluids from tumors [47–49]. High expression of serum exosomal miR-17-5p and miR-21 is observed in PDAC patients in association with metastasis and advanced stages [50]. These findings suggest that miR-100-5p liquid biopsy may be similarly applied to early detection of PDAC or postoperative recurrence monitoring in diabetic patients, especially those with poor glycemic control.

Diabetes therapy can influence the progression of PDAC [51]. Metformin can suppress tumor progression, while the effects of DPP4 inhibitors and insulin are still controversial [51–54]. In our study, treatment with metformin and a DPP4 inhibitor had no effects on the frequency of *CDH1* hypermethylation despite their hypoglycemic and anti-inflammatory effects. Although the reason is unclear, this might be ascribed to a statistical power issue due to the relatively small number of the subjects in this study. On the other hand, DPP4 inhibitor significantly increased the expression of miR-100-5p in PDAC. DPP4 inhibitors can modify various types of miRNA expression via activation of glucagon-like peptide-1 (GLP-1) signaling independent of glycemic improvement [55]. This suggests that miR-100-5p expression can be regulated by GLP-1 signaling in PDAC cells, which may lead to the progression of PDAC. Nevertheless, in the univariate analysis, patients treated with DPP4 inhibitor showed no inferior prognosis compared to patients not treated with DPP4 inhibitor. Because DPP4 inhibitors have moderate efficacy in controlling glucose levels and reduce HbA1c on average by approximately 0.6–0.8%, the improvement in blood glucose levels due to DPP4 inhibitor treatment may counteract the adverse prognostic effect elicited by the increase in miR-100-5p expression [56].

Our results suggest that strict glycemic control is necessary to suppress the expression of miR-100-5p in PDAC patients with DM. Because it is still unclear whether fasting blood glucose or glucose spikes affect miR-100-5p expression, further detailed investigation of blood glucose changes and miR-100-5p expression is required. In the case of PDAC with long-DM, concomitant administration of a demethylating agent such



	Median OS (days)	*p*-value
N-epi vs S-epi	1782 vs 979	< 0.05
N-epi vs D-epi	1782 vs 559	< 0.01
S-epi vs D-epi	979 vs 559	< 0.05

	Median DFS (days)	*p*-value
N-epi vs S-epi	1212 vs 406	< 0.05
N-epi vs D-epi	1212 vs 336	< 0.01
S-epi vs D-epi	406 vs 336	0.15

	Median OS (days)	*p*-value
long-DM/high-HbA1c vs Others	599 vs 1163	< 0.001

	Median DFS (days)	*p*-value
long-DM/high-HbA1c vs Others	341 vs 437	< 0.05

Figure 3. Survival curves based on OS and DFS. (A) The OS of the S-epi group (red line) was significantly lower than that of the N-epi group (black line). The survival rate of the D-epi group (green line) was significantly lower than that of the S-epi group ($p < 0.05$). (B) In terms of DFS, the survival rates of the S-epi and D-epi groups were significantly lower than that of the N-epi group ($p < 0.05$ and $p < 0.01$, respectively), while the survival rate of the S-epi group was comparable to that of the D-epi group. The group exhibiting both long-DM and high HbA1c showed significantly worse (C) OS and (D) DFS in subjects with PDAC associated with DM compared to the remaining diabetic subjects (others; $p < 0.001$ and $p < 0.05$, respectively). D-epi, subjects showing dual epigenetic change; N-epi, subjects showing no epigenetic change; S-epi, subjects showing a single epigenetic change.

as azacytidine is expected to restore *CDH1* gene expression by demethylating the promoter. Since azacytidine inevitably causes adverse events such as nausea, vomiting, and myelosuppression, DM history is considered useful in terms of patient selection for demethylating therapy [57].

There are several limitations to this study. First, only FFPE specimens were used. Since alteration of DNA during tissue processing is known to occur, the results may be confounded by technical artifacts. Although the quality of the samples was confirmed by immunostaining based on the reproducibility of the results in this study, confirmation of the results using fresh samples may be warranted in future investigations. Another drawback of this study may be that the prevalence of PDAC associated with DM was not directly evaluated because of the retrospective nature of the data. Future prospective studies will be necessary to confirm our results. Our analysis was also limited to *CDH1* promoter methylation and miR-100-5p, and it is impossible to speculate the whole scenario related to the implication of promoter methylation and miR expression in the development of PDAC based only on the findings for these two genes. Finally, it was not possible to evaluate epigenetic changes depending on the site of cancer tissue. Because the degrees of inflammatory cell infiltration and ischemia are different between the periphery and the center of cancer tissue, epigenetic traits may vary depending on the site. Future evaluation applying microdissection is required to address this.

Nevertheless, we believe that our study has revealed a novel association between E-cadherin downregulation, which can be mediated by multiple epigenetic changes, and DM in PDAC. Our results reconfirm the importance of optimal blood glucose control in DM. Future studies are expected to explore the possibility of developing new effective therapy applying demethylating agents for PDAC associated with DM.

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Author contributions statement

YH and HM conceived and designed the study. YH, HM and KH developed the methodology and wrote, reviewed and revised the paper. YH, KY, TY, AI, YT, TS, HK, KM, KK and KI acquired, analyzed and interpreted data and performed statistical analysis. HM and KH provided technical and material support. All authors read and approved the final paper.

Ethics approval and consent to participate

This study was approved by the ethical committee of Hirosaki University Graduate School of Medicine (#2020-143). This study was performed in accordance with the Declaration of Helsinki.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Preoperative HbA1c levels (within 1 month) showed a significant proportional correlation with average HbA1c levels 3–12 months before surgery in PDAC subjects

Table S1. Clinicopathological characteristics of 132 subjects

Table S2. Univariate analysis (disease-free survival)

Table S3. Multivariate analysis (disease-free survival)