Genome-wide DNA hypomethylation drives a more invasive pancreatic cancer phenotype and has predictive occult distant metastasis and prognosis potential

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Abstract. Genome-wide DNA hypomethylation is the most common molecular feature in human cancers associated with chromosomal instability (CIN), which is involved in the mechanisms that regulate pancreatic cancer (PC) metastasis. It was investigated whether genome-wide DNA hypomethylation affects the phenotype in PC via CIN *in vitro*, and its significance on the biological behavior of PC was verified. The relative demethylation level (RDL) of long interspersed nucleotide element-1 (LINE-1) in human PC cell lines was used to characterize DNA hypomethylation using methylation-specific quantitative (q)PCR. CIN was estimated by changes in chromosomal copy number using comparative genomic hybridization analysis. Abnormal segregation of chromosomes was assessed by immunocytochemistry, and the DNA damage response was evaluated using the number of anti-γH2AX positive cells. Invasion ability was assessed using a Matrigel invasion assay. Clinical specimens from 49 patients with PC who underwent curative surgery were evaluated for a correlation of DNA hypomethylation with clinical outcome. Successful induction of genome-wide DNA hypomethylation in PC cells led to copy number changes in specific chromosomal regions. The number of cells with abnormal segregation of chromosomes significantly increased with the number of anti-γH2AX positive cells. The invasive potential of these cells also significantly increased. The occurrence of occult distant metastasis in the clinical specimens and receiver operating characteristic analysis clearly identified those who were and were not likely to have occult distant metastasis, with high LINE-1 RDL significantly correlated with the presence of occult distant metastasis (P=0.035) and poor prognosis (P=0.048). The significance of genome-wide DNA hypomethylation on the biological behavior of PC, which promotes a more invasive phenotype via CIN *in vitro* and predicts the susceptibility to occult distant metastasis and poor prognosis in patients with PC was revealed.

Introduction

Pancreatic cancer (PC) is one of the most biologically aggressive solid organ tumors, with >70% of patients presenting with locally advanced or metastatic disease (1). Since PC is usually diagnosed at an advanced stage, only ~20% of patients qualify for initial resection (2,3). Even if curative resection is performed, most patients experience recurrence. The 5-year survival rate in patients undergoing complete resection is only ~25% (4). The molecular basis of this aggressive clinical behavior remains only partially clear (5-7). Surgical resection is the only potentially curative treatment for PC; however, a considerable proportion of patients undergo unnecessary laparotomy due to the underestimation of the extent of the cancer during preoperative radiographic examinations (8). Imaging modalities have poor sensitivity for identifying small liver or peritoneal metastases (9,10). A proportion (40%) of patients who undergo surgical exploration have tumors that are unresectable due to occult distant metastasis, which cannot be identified during preoperative examinations and is only discovered intraoperatively or by infiltration of local structures (8,11-14). For patients with distant occult metastasis, surgical resection does not prolong survival in the majority of patients (11,15,16). Therefore, it is critical to identify patients with PC who are likely to have occult distant metastasis to avoid unnecessary surgery and offer tailored treatments in a

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Abbreviations: PC, pancreatic cancer; CIN, chromosomal instability; LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level; 5-Aza-dC, 5-aza-2'-deoxycytidine; CGH, comparative genomic hybridization; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; DUPAN-2, duke pancreatic monoclonal antigen type 2; OS, overall survival

timely manner. Although there have been reports on the correlation between clinical factors such as preoperative tumor marker levels and tumor size and the presence of occult distant metastasis, these biomarkers have not yet been recognized as useful biomarkers (1,11,12).

Epigenetic alterations are among the most common features of human cancers and are associated with cancer development and progression (17,18). A major epigenetic alteration is DNA methylation abnormality, which is the post-replicative addition of a methyl group to the fifth carbon of the cytosine ring in CpG dinucleotides. DNA methylation alterations of CpG islands in gene promoter regions lead to transcriptional suppression of tumor suppressor genes, which are involved in multistep carcinogenesis. Genome-wide DNA hypomethylation is another type of epigenetic alteration that is mainly caused by the demethylation of DNA repetitive sequences that are normally methylated. Repetitive DNA sequences are widely distributed in the human genome and are ideal targets for DNA hypomethylation. These sequences are involved in tumor progression in ovarian epithelial and hepatocellular carcinomas (19,20). Long interspersed nucleotide element-1 (LINE-1) is a transposable element in the human genome that comprises a repetitive sequence and constitutes a substantial proportion (~17%) of the human genome (21-24). The level of LINE-1 methylation is associated with genome-wide DNA methylation (24-27) and with poor prognosis in various human cancers (27,28).

Chromosomal instability (CIN) is a major driver of tumor evolution and a hallmark of cancer, that results from ongoing errors in chromosome segregation during mitosis (29,30). Experimental studies using mouse models have provided evidence that genome-wide DNA hypomethylation induces CIN (17,31). The development of CIN has been reported to be correlated with tumor metastasis (32,33). Additionally, LINE-1 hypomethylation has been reported to be significantly associated with CIN in gastrointestinal stromal tumors (34). Indeed, it was previously demonstrated that DNA hypomethylation and its connection with DNA copy number changes is associated with a poor prognosis in colon and stomach cancers (35,36). Similarly, overexpression of repetitive sequences induced by retrovirus-expressing vectors leads to changes in copy number at specific chromosomes (37). These data suggested that genome-wide DNA hypomethylation drives the cancer phenotype to be more invasive by affecting the machinery that maintains chromosomal stability. However, no study has been conducted to elucidate the impact of genome-wide DNA hypomethylation on the biological behavior of PC in connection with CIN.

In the present study, it was investigated whether genome-wide DNA hypomethylation promotes the invasive ability and metastatic potential via CIN in human PC cell lines. The significance of genome-wide DNA hypomethylation on the biological behavior of PC and their correlation with clinical outcomes were also verified.

Materials and methods

Cell lines and cell culture. The human PC cell lines PANC-1 and Capan-1 were obtained from the American Type Culture Collection. Cultured PANC-1 cells were maintained in RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (FBS; Cytiva) and Capan-1 cell was cultured in IMDM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 20% FBS at 37˚C in a humidified atmosphere containing 5% CO_2.

Clinical samples. A total of 49 clinical samples were collected from patients with PC who underwent surgery between September 2010 and July 2017 at Saitama Medical Center of Jichi Medical University (Saitama, Japan). No metastasis was diagnosed based on preoperative imaging studies or intended curative surgery. The patients did not undergo adjuvant chemotherapy before surgery. Clinical data were collected by a review of patient medical records. The preoperative variables included sex, age, smoking, alcoholic consumption, serum carcinoembryonic antigen (CEA) content, serum carbohydrate antigen 19-9 (CA19-9) content, serum duke pancreatic monoclonal antigen type-2 content, tumor size, tumor location and clinical stage classified with the Union for International Cancer Control 7th edition (38). Intraoperative variables that were recorded included the presence of distant metastases such as liver or peritoneal metastases, and were defined as occult distant metastasis. Overall survival (OS) in these patients was calculated as the time from surgery to the occurrence of the event. Additional clinical samples from 5 patients with stage IV were collected to compare their metastatic potential with that of 49 patients. These 5 patients did not undergo curative surgery, and one patient had received chemotherapy before clinical samples were collected. The study protocol was approved (approval no. 21-09) by the research ethics committee of Jichi Medical University (Saitama, Japan) and conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki (seventh revision).

Treatment with 5-aza-2'-deoxycytidine (5-Aza-dC). The demethylating agent 5-Aza-dC was purchased from FUJIFILM Wako Pure Chemical Corporation. The drug was dissolved in phosphate-buffered saline as a 1 mM stock solution, passed through a 0.22-μm filter, and stored at -20˚C in aliquots that were thawed immediately prior to use. The stock was diluted in cell culture medium at different concentrations and was applied to cells 24 h after cell seeding. Fresh medium supplemented with 5-Aza-dC was replaced every 24 h. Cultures without 5-Aza-dC were used as the control group.

MTT cell viability assay. PANC-1 and Capan-1 cells were seeded at a density of 5,000 cells/well in 96-well plates. A total of 24 h after seeding, cells were treated with 0.1, 1, 5, 10, and 20 μM 5-Aza-dC, with fresh 5-Aza-dC solutions replaced every 24 h. Assays were conducted on extracts harvested every 24 h for 1 to 7 days after the beginning of the 5-Aza-dC treatment. Cell viability was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric dye reduction using the MTT assay kit (TOX-1; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. After the MTT reagent was added, the cells were incubated for 4 h at 37˚C, after which the solubilization solution (10% Triton X-100, 0.1 N HCl and anhydrous isopropanol) was added and absorbance

Table I. PCR primers and TaqMan probes used in the MethyLight method.

Alu-C	F: GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA
	R: ATTAACTAAACTAATCTTAAACTCCTAACCTCA
	Probe: FAM-CCTACCTTAACCTCCC-MGB
LINE-1	F: TTTATTAGGGAGTGTTAGATAGTGGGTG
	R: CCTTACACTTCCCAAATAAAACAATACC
	Probe: FAM-CACCCTACTTCAACTCATACACAATACACACACCC-MGB

PCR, polymerase chain reaction; LINE-1, long interspersed nucleotide element-1.

was measured using a microplate reader (Varioskan LUX multimode reader; Thermo Fisher Scientific, Inc.) at a wavelength of 570 nm. Background absorbance at 690 nm was subtracted from the 570 nm measurement, and the percentage of viable cells was determined relative to the control. Each experiment was performed in triplicate for each treatment condition.

DNA extraction and bisulfite modification. Genomic DNA was isolated and purified from the cultured cells treated or without 5-Aza-dC using an EZ1 Advanced XL (Qiagen GmbH), and from clinical samples using the QIAamp DNA FFPE Tissue kit (Qiagen GmbH) according to the manufacturer's instructions. DNA purity was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) at absorbances of 260 and 280 nm. In all instances, the A260/A280 ratio exceeded 1.8. Sodium bisulfite conversion of genomic DNA was performed using an EpiTect Bisulfite kit (Qiagen GmbH). DNA quantities of 100 ng in a volume of up to 40 μl were processed using this standard protocol. The treatment of genomic DNA with sodium bisulfite converted unmethylated cytosine to uracil, which was then converted to thymidine during subsequent PCR steps. This process revealed sequence differences between methylated and unmethylated DNA.

MethyLight methods. Following bisulfite modification, each sample was examined using MethyLight technology for the LINE-1 sequence. Two sets of primers and probes specifically designed to bind to bisulfite-converted DNA were used in the reaction: one set of LINE-1 primers and a probe for unmethylated target analyses (unmethylated reaction) and another set of primers for the reference locus, ALU-C (normalization control reaction), as previously described (26,37,39). The primer and probe sequences are summarized in Table I. Whole-genome amplification provided fully unmethylated DNA obtained from human genomic DNA (Promega Corporation), which served as the demethylation constant reference that enabled determination of the relative demethylation level (RDL). The LINE-1 RDL was defined as (LINE-1 reaction/ALU-C reaction) sample/(LINE-1 reaction/ALU-C reaction) fully unmethylated control DNA, as previously described (37,39). In each MethyLight reaction, 1 μl bisulfite-modified DNA solution was used. Thermal cycling was initiated with a denaturation step at 95˚C for 10 sec, followed by 50 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Real-time quantitative PCR (40) was performed on a QuantStudio 12 K Flex Real-Time PCR

System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a final reaction volume of 20 μl containing Premix Ex Taq (Takara Bio, Inc.), 600 nM of each primer and 200 nM probe. Table SI shows the Ct (cycle threshold) values and their variability in the different reactions in the present study.

Microarray-based comparative genomic hybridization (array CGH). Array CGH was performed using a SurePrint G3 Human CGH Microarray kit, 8x60 K (Agilent Technologies, Inc.). Labeling and hybridization were performed using a SureTag DNA Labeling kit and Oligo aCGH/ChiP-on-chip hybridization kit (both from Agilent Technologies, Inc.) according to the manufacturer's protocol (Protocol v8.0). In brief, ≥0.2 μg DNA from the samples and an equal amount of control DNA were digested with AluI and RsaI for 2 h at 37˚C. The digested DNA was labeled by random priming and then the sample and control DNA were labeled with Cy5-dUTP and Cy3-dUTP, respectively. The labeled products were purified using an Amicon Ultra-0.5 Centrifugal Filter Unit with an Ultracel-30 membrane (MilliporeSigma) and concentrated to 9.5 μl. Dye incorporation and DNA yield were measured using a NanoDrop ND-1000 spectrophotometer. Equal amounts of genomic DNA extracted from samples and control DNA were mixed with human Cot-1 DNA and then dissolved in hybridization buffer (Agilent Technologies, Inc.), denatured, and hybridized to the CGH array at 67˚C for 24 h. Following hybridization, the microarrays were washed with Oligo aCGH/ChIP-on-Chip Wash Buffer (Agilent Technologies, Inc.). After washing, the slides were scanned using an Agilent Technologies Microarray Scanner. Microarray images were analyzed using a feature extraction software program v.12.0.3.1 (Agilent Technologies, Inc.), and the resulting data were subsequently imported into the Agilent Cytogenomics software program, v5.1.

Immunocytochemistry. Cells were cultured on cover glass in 24-well plates. A total of 24 h after seeding, the cells were treated with 400 ng/ml nocodazole (product code ab120630; Abcam) for 18 h to synchronize the cell cycle with the mitotic phase as previously described (41-43). A total of 40 min after release from nocodazole arrest, the prepared cover glass samples were fixed in 4% paraformaldehyde/PBS at room temperature for 10 min and then washed three times with PBS. The cells were permeabilized with 0.1% Triton X-100 (Agilent Technologies, Inc.) at room temperature for 10 min and blocked with 10% normal goat serum (Thermo Fisher Scientific, Inc.) at room temperature for 30 min. The cells were washed three

times with PBS and incubated with anti-α-tubulin (1:500; product code ab7291) and anti-γH2AX (1:1,000; product code ab11174; both from Abcam) at 4°C overnight. Following extensive washing with PBS, the samples were incubated with Alexa-594-conjugated anti-mouse IgG secondary antibody (1:200; product code ab150120) and Alexa-488-conjugated antirabbit IgG secondary antibody (1:200; product code ab150081; both from Abcam) for nuclear staining at room temperature for 60 min. The cover glass samples were washed three times with PBS, mounted in VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (cat. no. H-1800; Vector Laboratories, Inc.), and sealed with nail polish. Images were acquired using a Keyence BZ-X700 fluorescence micro-scope (Keyence Corporation).

Invasion assay. The invasion ability of PC cells was assessed using a Corning BioCoat Matrigel Invasion Chamber assay (Corning® 354480; Corning, Inc.) according to the manu-facturer's protocol. After PANC-1 and Capan-1 cells were treated with 1 μM 5-Aza-dC for 3 days, they were seeded at 25,000 cells/well in serum-free medium in the upper chambers of 24-well plates. A culture medium containing 10% FBS was used in the lower chamber as a chemoattractant. After incubation for 22 h at 37°C, the medium was removed. After the removal of the non-migratory cells, the cells in the upper chamber were fixed and permeabilized for 2 min at room temperature, and stained for 4 min at room temperature using a Diff-Quick® simple and rapid staining solution (Sysmex Corporation) according to the manufacturer's protocol. A total of 5 random fields per well in four independent experiments were observed using a Keyence BZ-X700 fluorescence microscope (Keyence Corporation) to assess cell migration. Captured images were analyzed using ImageJ software (v. 1.52a; National Institutes of Health). The invasion ability of PC cells was evaluated as percentage of invasion and was calculated by the following formula: Percentage of invasion=(average number of cells invading Matrigel coated membrane)/(average number of cells migrating through control (no Matrigel coating) membrane) x100, according to the manufacturer's instructions.

Statistical analyses. All statistical analyses were performed using EZR version 1.41 (Saitama Medical Center; Jichi Medical University), which is a graphical user interface for R version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria) (44). Continuous variables were evaluated using the Shapiro-Wilk normality test, and the means or medians were then compared with the paired samples t-test for normally distributed variables, or the Mann-Whitney U-test and Kruskal-Wallis test for non-normally distributed variables. Logistic regression analysis was used to examine the associations between two categorical variables. The correlation between the two variables was evaluated using the Spearman's rank correlation coefficient. The survival time distribution was assessed using the Kaplan-Meier method with a log-rank test. A Cox proportional hazards regression model was used to evaluate the association between overall mortality and other factors in univariate and multivariate analyses. All reported P-values were two-sided, and P-values <0.05 were considered to indicate a statistically significant difference.

Figure 1. Cell viability of human pancreatic cancer cell lines treated with 5-Aza-dC. (A and B) Time and dose-dependent cytotoxicity of 5-Aza-dC was evaluated in the two human pancreatic cancer cell lines: (A) PANC-1 and (B) Capan-1. Cell viability was assessed throughout the time course using an MTT assay. The data are presented as the mean ± standard devia-tion and are representative of three independent experiments. 5-Aza-dC, 5-aza-2'-deoxycytidine.

Figure 2. Induction of DNA hypomethylation in human pancreatic cancer cell lines treated with 5-Aza-dC. Increased levels of DNA hypomethylation were determined using the RDL of LINE-1 in (A) PANC-1 and (B) Capan-1 cell lines that were treated with 1 μM 5-Aza-dC for 3 days when compared with untreated PANC-1 and Capan-1 cell cultures, respectively. The data are representative of 4 independent experiments. ****P<0.0001. LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level; 5-Aza-dC, 5-aza-2'-deoxycytidine.

Results

Viability of PANC-1 and Capan-1 cells treated with 5-Aza-dC. First, the optimal concentration of 5-Aza-dC and the dura-tion of treatment for the PC cell lines were determined. The MTT cell viability assays showed that cell viability gradually decreased in a dose-dependent manner after treatment with

Figure 3. Abnormal segregation of chromosomes, DNA damage and chromosomal instability in response to 5-Aza-dC treatment in pancreatic cancer cell lines. (A) The number of cells with abnormal segregation in PANC-1 cells increased after treatment with 1 μM 5-Aza-dC for 3 days when compared with untreated controls. (B-E) Representative images of abnormal segregation including (B) micronuclei, (C) lagging, (D) anaphase bridge and (E) multiple nuclei in PANC-1 cells treated with 5-Aza-dC (Scale bar, 10 μm). (F and G) Increased number of cells with DNA damage in (F) PANC-1 and (G) Capan-1 cell lines treated with 1 μM 5-Aza-dC for 3 days compared with the untreated controls. DNA damage was evaluated by the number of anti-γH2AX positive cells. (H) Images of anti-γH2AX positive cells (Scale bar, 20 μm). (I and J) Copy number changes in chromosomes detected by CGH array in (I) PANC-1 and (J) Capan-1 cells treated with 1 μM 5-Aza-dC for 3 days. Copy number changes were observed more in PANC-1 cells when compared with Capan-1 cells. Gains are shown in the upper area and losses in the lower area. Numbers represent the chromosome number. In each chromosome area, the short arm is located on the left side, and the long arm is on the right side. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. 5-Aza-dC, 5-aza-2'-deoxycytidine; CGH, comparative genomic hybridization.

1 μM 5-Aza-dC for 3 days; however, doses of 5-Aza-dC greater than 1 μM or treatment periods longer than 3 days led to significant cytotoxicity (Fig. 1A and B and Table SII). Therefore, 5-Aza-dC was used at a concentration of 1 μM for 3 days in the present study.

5-Aza-dC enhancement of DNA hypomethylation. It was next verified whether 5-Aza-dC induced DNA hypomethylation in PC cell lines. PANC-1 and Capan-1 cells were cultured with or without 1 μM 5-Aza-dC for 3 days before the RDL of the LINE-1 repetitive sequence was used to determine the level of genome-wide DNA hypomethylation (24-27). LINE-1 RDLs in PANC-1 and Capan-1 cells were significantly elevated after treatment with 5-Aza-dC (PANC-1, 0.19±0.097 to 0.44±0.067, P=0.00002; Capan-1, 0.28±0.024 to 0.53±0.066, P=0.000005; Fig. 2A and B). These results

Table II. Association between clinical features and LINE-1 RDLs of patients with pancreatic cancer.

Clinical features	Total n (%)	LINE-1 RDL (median)	P-value
All cases	49 (100)	0.045	
Age, years			0.21
<70	24 (41.0)	0.047	
≥70	25 (49.0)	0.045	
Sex			0.49
Male	25 (49.0)	0.046	
Female	24 (41.0)	0.045	
Smoking			0.41
No	24 (41.0)	0.045	
Yes	25 (49.0)	0.049	
Alcohol consumption			0.24
No	41 (83.7)	0.045	
Yes[a]	8 (16.3)	0.045	
CEA (ng/ml)			0.058
≤5	39 (79.6)	0.044	
>5	10 (20.4)	0.063	
CA19-9 (U/ml)			0.72
≤37	11 (22.4)	0.044	
>37	38 (77.6)	0.046	
DUPAN-2 (U/ml)			0.12
≤150	28 (57.1)[b]	0.044	
>150	19 (38.8)	0.055	
Tumor size (cm)			0.0011
≤2	18 (36.7)	0.037	
>2	31 (63.3)	0.052	
Clinical stage[c]			0.045
I	15 (30.6)	0.040	
II	33 (67.3)	0.052	
III	1 (2.0)	0.043	
IV	0	NA	
Tumor location			0.42
Head	28 (57.1)	0.048	
Body	11 (22.4)	0.040	
Tail	10 (20.4)	0.045	
Occult distant metastasis			0.034
Absent	42 (86)	0.045	
Present	7 (14)	0.062	

[a]Intake ≥30 g/day. [b]No data were available for two cases. [c]Clinical stage was classified by the Union for International Cancer Control. CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; DUPAN-2, duke pancreatic monoclonal antigen type 2; NA, not available; LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level.

Figure 4. Change in the invasion ability of human pancreatic cancer cells after treatment with 5-Aza-dC. Invasion ability was enhanced in (A) PANC-1 and (B) Capan-1 cells after treatment with 1 μM 5-Aza-dC for 3 days. The invasion ability was determined with a Matrigel invasion assay. The data are representative of 4 independent experiments. Data are presented as the mean ± SD. (C and D) Representative images of cells invading through the Matrigel-coated membrane in invasion assay of (C) PANC-1 and (D) Capan-1 cell lines with and without 5-Aza-dC treatment. The small circles are 8-μm membrane pores and cells invading the Matrigel basement membrane matrix and pores are stained blue (Scale bar, 20 μm). *P<0.05. 5-Aza-dC, 5-aza-2'-deoxycytidine.

indicated that the 5-Aza-dC treatment could induce DNA hypomethylation in PC cells.

Abnormal segregation of chromosomes, DNA damage and chromosomal instability in response to 5-Aza-dC treatment. Immunocytochemistry was performed to detect abnormal segregation of chromosomes and DNA damage, which could lead to CIN. DNA damage response was evaluated using the number of anti-γH2AX positive cells. The number of cells with abnormal segregation of chromosomes including micronuclei, lagging, anaphase bridging, and multiple nuclei significantly increased (3.9±2.7 to 12.5±3.6%, P=0.0005; Fig. 3A-E), with elevated numbers of anti-γH2AX positive cells (15.1±3.2 to 55.6±7.9%, P=0.028; Fig. 3F) in PANC-1 cells treated with 5-Aza-dC when compared with the untreated control. In Capan-1 cells, the nuclei were fused with each other, making it difficult to identify abnormal segregations; however, the number of anti-γH2AX positive cells significantly increased (47.8±8.4 to 58.9±8.8%, P=0.0032; Fig. 3G). Fig. 3H demonstrates representative images of anti-γH2AX positive cells. The incidences of abnormal segregation and anti-γH2AX positive cells were investigated in more than 100 mitotic cells per

Table III. Univariate and multivariate analyses of presenting occult distant metastasis in patients with pancreatic cancer.

Clinical features	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Age (<70 vs. ≥70)	0.68 (0.14-3.43)	0.64		
Sex (male vs. female)	0.68 (0.14-3.43)	0.64		
Smoking (no vs. yes)	1.33 (0.27-6.70)	0.73		
Alcohol consumption (no vs. yes)	2.40 (0.38-15.3)	0.36		
CEA (ng/ml) (≤5 vs. >5)	1.70 (0.28-10.4)	0.57	0.39 (0.033-4.59)	0.46
CA19-9 (U/ml) (≤37 vs. >37)	2.61e+07 (0-Inf)	0.99	2.33e+08 (0-Inf)	0.99
DUPAN-2 (U/ml) (≤150 vs. >150)	4.64 (0.8-27.1)	0.088	5.07 (0.43-59.8)	0.20
Tumor size (cm) (≤2 vs. >2)	4.08 (0.45-37.0)	0.21	5.71 (0.16-208)	0.34
Clinical stage[a] (I vs. II, III)	3.0 (0.33-27.4)	0.33	0.057 (0.00063-5.14)	0.21
Tumor location (head vs. body, tail)	0.48 (0.084-2.78)	0.42	0.62 (0.069-5.51)	0.66
LINE-1 RDL[b] (<0.049 vs. ≥0.049)	10.8 (1.19-98.4)	0.035	22.2 (1.06-464)	0.046

[a]Clinical stage was classified by the Union for International Cancer Control. [b]LINE-1 RDL cut-off value 0.049 was determined by Receiver operating characteristic analysis for correlation between LINE-1 RDL and the presence of occult distant metastasis (Fig. 5A). OR, odds ratio; CI, confidence interval; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; DUPAN-2, duke pancreatic monoclonal antigen type 2; LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level.

Figure 5. Correlation between LINE-1 RDL and occult distant metastasis in clinical pancreatic cancer samples. (A) Receiver operating characteristics analysis determined a LINE-1 RDL of 0.049 as the cut-off value for identifying patients with occult distant metastasis. (B) Distribution of the LINE-1 RDL in 49 tumor specimens in increasing order. The black and grey boxes indicate tumors with and without occult distant metastasis, respectively. The dotted line indicates the cut-off value of 0.049. LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level.

sample and were representative of three distinct experiments. Changes in copy number were analyzed using CGH array analysis. Successful induction of DNA hypomethylation led to copy number changes in specific regions of the chromosomes in PC cells after treatment with 5-Aza-dC (Fig. 3I and J), with copy number changes being more likely to occur in PANC-1 cells than in Capan-1 cells. These results suggested that 5-Aza-dC induced DNA hypomethylation that led to CIN in human PC cells.

Invasion ability of PC cells after 5-Aza-dC treatment. The invasion ability of PANC-1 and Capan-1 PC cells was assessed after treatment with 5-Aza-dC using the Matrigel invasion assay. The number of cells that migrated through the membrane represented the invasive and metastatic potential of the cancer cells. Before 5-Aza-dC treatment, Capan-1 cells migrated through the membrane more than twice as much as PANC-1 (PANC-1, 16.6±11.9%; Capan-1, 35.4±11.4%; Fig. 4A and B), indicating that Capan-1 cells harbored more invasive potential than PANC-1 cells. After 5-Aza-dC treatment, the number of PANC-1 and Capan-1 cells that migrated increased significantly compared with the untreated cells, respectively (PANC-1, 16.6±11.9 to 37.9±8.0%, P=0.042; Capan-1, 35.4±11.4 to 66.5±9.7%; P=0.011; Fig. 4A and B). Representative images of cells invading through the Matrigel-coated membrane of PANC-1 and Capan-1 cell lines with and without 5-Aza-dC

Table IV. Univariate and multivariate analyses of overall survival in patients with pancreatic cancer.

Prognostic factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (<70 vs. ≥70)	1.13 (0.53-2.40)	0.76		
Sex (male vs. female)	0.90 (0.42-1.94)	0.79		
Smoking (no vs. yes)	1.00 (0.47-2.14)	1.00		
Alcohol consumption (no vs. yes)	0.83 (0.25-2.77)	0.77		
CEA (ng/ml) (≤5 vs. >5)	0.60 (0.21-1.76)	0.53	0.33 (0.076-1.39)	0.13
CA19-9 (U/ml) (≤37 vs. >37)	1.45 (0.55-3.84)	0.45	2.03 (0.66-6.26)	0.22
DUPAN-2 (U/ml) (≤150 vs. >150)	1.03 (0.47-2.28)	0.93	1.04 (0.39-2.77)	0.93
Tumor size (cm) (≤2 vs. >2)	0.79 (0.37-1.68)	0.54	0.88 (0.32-2.39)	0.80
Clinical stage[a] (I vs. II, III)	0.85 (0.38-1.90)	0.69	0.48 (0.15-1.59)	0.23
Tumor location (head vs. body, tail)	0.68 (0.32-1.48)	0.33	0.49 (0.21-1.15)	0.099
LINE-1 RDL[b] (<0.049 vs. ≥0.049)	1.80 (0.84-3.85)	0.13	3.40 (1.23-9.38)	0.018

[a]Clinical stage was classified by the Union for International Cancer Control. [b]LINE-1 RDL cut-off value 0.049 was determined by Receiver operating characteristic analysis for correlation between LINE-1 RDL and the presence of occult distant metastasis (Fig. 5A). HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; DUPAN-2, duke pancreatic monoclonal antigen type 2; LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level.

treatment are presented in Fig. 4C and D. Cells treated with 5-Aza-dC appeared to be more invasive than untreated cells. Notably, the increased number of migratory PANC-1 cells after 5-Aza-dC treatment (37.9±8.0%) was the same as that of naïve Capan-1 before treatment (35.4±11.4%), which indicated that PANC-1 cells acquired an invasive potential similar to Capan-1 cells, further suggesting that genome-wide DNA hypomethylation-induced CIN drives the PC phenotype to be more invasive.

Clinical outcomes and LINE-1 RDL in patients with PC undergoing curative surgery. The significance of genome-wide DNA hypomethylation in the biological behavior of PC in 49 patients who underwent curative surgery was investigated. The clinical features of the 49 patients and their relation to the LINE-1 RDL of the tumor specimens are shown in Table II. The mean age of all patients was 68 years, and the median age was 70 (range, 40-83) years. This median age was used as the boundary for analysis in the present study. Occult distant metastasis was found in 7 of the 49 patients (14.3%), although all patients were diagnosed without metastasis by imaging studies performed before surgery. Significant correlation of the LINE-1 RDL was observed with tumor size (P=0.0012), clinical stage (P=0.045) and the presence of occult distant metastasis (P=0.034). The presence of occult distant metastasis and its relationship to the LINE-1 RDL were then addressed by performing receiver operating characteristic analysis to determine the cut-off value of the LINE-1 RDL to identify patients with occult distant metastasis (Fig. 5A). The area under the curve was 0.76 (95% confidence interval 0.58-0.93) and the derived cut-off value of the LINE-1 RDL was 0.049 with a sensitivity and specificity of 0.86 and 0.64, respectively. The distribution of the LINE-1 RDL in 49 tumor specimens in increasing order of LINE-1 RDL is presented in Fig. 5B. A total of 6 out of 21 patients with high LINE-1

RDL ≥0.049 had occult distant metastasis, whereas only one in 28 patients with low LINE-1 RDL <0.049 displayed occult distant metastasis. Univariate and multivariate analyses revealed that LINE-1 RDL was a significant independent predictor of occult distant metastasis (Table III). In addition, the impact of LINE-1 RDL on patient prognosis was verified. Correlation coefficient analysis revealed a significant correlation between LINE-1 RDL and OS in 49 patients (rho=-0.37, P=0.0089; Fig. 6A), while multivariate analysis showed that LINE-1 RDL was a significant independent prognostic factor (Table IV). However, it was not possible to demonstrate a significant difference in OS between patients with high and low LINE-1 RDL using Kaplan-Meier analysis, although patients with high LINE-1 RDL tended to have worse OS than those with low LINE-1 RDL (median OS, 31.9 M vs. 57.7 M; P=0.13; Fig. 6B). The modified LINE-1 RDL of 0.045 was then applied as a median value to Kaplan-Meier analysis, which demonstrated that patients with high LINE-1 RDL had a significantly worse OS than those with low LINE-1 RDL (median OS, 27.2 M vs. not reached; P=0.048; Fig. 6C). In addition, LINE-1 RDL was determined in five additional patients diagnosed with clinical stage IV and significantly higher LINE-1 RDL at 0.052, 0.082, 0.113, 0.230 and 0.331 was confirmed, respectively (data not shown).

Discussion

To the best of our knowledge, the present study is the first to show that genome-wide DNA hypomethylation induced by treatment with 5-Aza-dC can drive the invasive phenotype of PC via CIN in PC cells. Furthermore, by using LINE-1 RDL, the extent of genome-wide DNA hypomethylation that is involved in the aggressive behavior of PC, such as occult distant metastasis and poor prognosis, was determined. These findings suggested that LINE-1 RDL is a

Figure 6. Correlation between LINE-1 RDL and OS in patients with pancreatic cancer. (A) Significant correlation between LINE-1 RDL and OS was reported in 49 pancreatic cancer patients using correlation coefficient analysis (rho=-0.37, P=0.0089). (B) Kaplan-Meier analysis did not show a significant difference in OS between patients with high and low LINE-1 RDL using the 0.049 cut-off value (median OS; 31.9 M vs. 57.7 M, P=0.13). (C) Using a modified LINE-1 RDL of 0.045, Kaplan-Meier analysis demonstrated that 49 patients with a high median LINE-1 RDL had significantly worse OS than those with low LINE-1 RDL (median OS; 27.2 M vs. not reached, P=0.048). OS, overall survival; LINE-1, long interspersed nucleotide element-1; RDL, relative demethylation level; CI, confidence interval.

potent epigenetic biomarker for the selection of patients with PC with occult distant metastases who are candidates for curative surgery.

In the present study, genome-wide DNA hypomethylation was induced in human PC cell lines with 5-Aza-dC treatment. A significant increase in DNA hypomethylation was observed in both cell lines regardless of their invasive potential. In a previous study, DNA hypomethylation was successfully induced in colon cancer cell lines with 5-Aza-dC treatment (45). Induction of DNA hypomethylation was verified by methylation-sensitive amplified fragment-length polymorphism, which is a fingerprinting technique that simultaneously analyzes DNA methylation in hundreds of loci (45,46). Genome-wide DNA hypomethylation was reported to be induced in several tumors after treatment with 5-Aza-dC and is widely assessed by LINE-1 (18,47-50). Consistent with these findings, it was reported that genome-wide DNA hypomethylation was induced in PC cell lines.

Since the experimental mouse model has been used to show that genome-wide DNA hypomethylation induces CIN (17,31), it has also been used to investigate aneuploidy of chromosomes. The successful induction of genome-wide DNA hypomethylation in the present study led to copy number changes in specific regions of chromosomes that was concomitant with mitotic chromosomal errors and DNA damage in PC cell lines, which was consistent with the report that aneuploidy of chromosomes increased when genome-wide DNA hypomethylation was induced in colon cancer cells treated with 5-Aza-dC (18). However, no other studies have clarified the relationship between genome-wide DNA hypomethylation and CIN in PC cells after 5-Aza-dC treatment. Furthermore, the present study demonstrated that changes in copy number were more likely to be observed in PANC-1 than in Capan-1 cells, suggesting that cells that do not have invasive potential are more susceptible to CIN induced by genome-wide DNA hypomethylation.

CIN is an evolutionary process of PC metastasis (32-34) and poor patient prognosis. Bakhoum et al (51) showed that CIN promotes metastasis by sustaining a tumor-cell autonomous response to cytosolic DNA in breast cancer. In the present study, the number of migratory cells in PANC-1 cells treated with 5-Aza-dC (37.9±8.0%) was the same as that in naïve Capan-1 cells before treatment (35.4±11.4%), indicating that PANC-1 acquired invasive potential similar to Capan-1 (Fig. 4) and that genome-wide DNA hypomethylation affects the invasive nature of the PC phenotype through the induction of CIN.

Metastasis is the most common cause of cancer-related death in patients with cancer in clinical practice, particularly in patients with PC that have a poor prognosis. Identification of markers that can successfully predict occult distant metastasis is critical for improving patient prognosis and identifying appropriate candidates for curative surgery. Liu et al (11) have reported that younger age, male sex, larger tumor size, low serum ALT level, and high serum CA19-9 level are independent predictors of unexpected distant metastasis on exploration. Although certain studies have suggested a role for tumor location, serum CEA level, and clinical findings such as weight loss and jaundice, currently insufficient evidence exists for the applicability of these variables in predicting resectability (1,12). The present study demonstrated that there was a significant correlation before the LINE-1 RDL and clinical

variables such as tumor size, clinical stage and the presence of occult distant metastasis. The significance of genome-wide DNA hypomethylation was then elucidated using LINE-1 RDL as a predictor of occult distant metastasis. Occult distant metastasis was recognized in six out of 21 patients with high LINE-1 RDL ≥0.049, although these patients were diagnosed to not show metastasis by imaging studies performed before surgery. The analysis of LINE-1 RDL facilitates the identification of patients likely to have occult distant metastasis, who require further exploration for unresectability before surgery. De Rosa *et al* (12) have reported that serum CA19-9 level and tumor size are potential predictors of unresectability in selecting patients for staging laparoscopy. By contrast, only one in 28 patients with low LINE-1 RDL <0.049 displayed occult distant metastasis. These patients with a low risk of occult distant metastasis, as assessed by LINE-1 RDL, would be appropriate candidates for curative surgery. Total assessment of LINE-1 RDL in combination with serum CA19-9 level and tumor size would be reliable surrogate markers for selecting patients for curative surgery without staging laparoscopy before operation.

Previous studies have reported that LINE-1 hypomethylation is associated with clinical outcome in patients with several types of cancer (27,28). In the present study, a significant correlation was also observed between LINE-1 RDL and OS in patients with PC, with multivariate analysis indicating that LINE-1 RDL is a significant independent predictor of prognosis (Table IV). Yamamura *et al* (28) reported no significant association between LINE-1 hypomethylation and prognosis in patients with PC using a cohort that included 10% of patients with stage IV PC, which may have affected prognosis. Patients with stage IV disease were excluded and focus was addressed on patients who underwent curative surgery without extensive invasion or distant metastasis. Although no significant difference in OS was identified between patients with high and low LINE-1 RDL in the present study, patients with high LINE-1 RDL tended to have a worse OS than those with low LINE-1 RDL. A modified LINE-1 RDL of 0.045 revealed a significant difference in OS between patients with high and low LINE-1 RDL. The difference in patient selection using LINE-1 hypomethylation may affect its association of clinical outcomes between the present study and that of Yamamura *et al* (28).

The present study had certain limitations. The optimal concentration of 5-Aza-dC to prevent cell death in PC cell lines was determined, but the possibility that the cytotoxicity induced by 5-Aza-dC was associated with the induction of CIN could not be excluded. Furthermore, 5-Aza-dC may alter the transcription of multiple genes that could potentially affect chromosomal segregation fidelity and invasiveness. In addition, CIN was induced by genome-wide DNA hypomethylation following treatment with 5-Aza-dC, but it is unknown which was more responsible for enhancing the invasive potential of the treated cells. Further studies with a larger and more inclusive patient cohort are also required to confirm the significance of LINE-1 hypomethylation as a potent epigenetic biomarker in the prognosis of patients with stage IV cancer in clinical practice.

In conclusion, the present findings indicated that successful induction of CIN by genome-wide DNA hypomethylation induces the phenotype of PC to become more invasive *in vitro*, and that genome-wide DNA hypomethylation is involved in the biological behavior of PC, such as occult distant metastasis and poor patient prognosis in PC. LINE-1 RDL could be a potent epigenetic biomarker for the selection of patients who could benefit from staging laparoscopy to avoid unnecessary laparotomy and develop bespoke treatments in a timely manner. It is considered that the present data shed light on new strategies for treating patients with PC who could benefit from curative surgery.

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Availability of data and materials

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

Authors' contributions

YE designed the study and wrote the initial draft of the manuscript. KS contributed to the analysis and interpretation of the data and assisted in the preparation of the manuscript. YK, ST, HA, IA, FW, TK, MS, KF, HN, FK and TR contributed to data collection and interpretation, and critically reviewed the manuscript. YE and KS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Jichi Medical University (Saitama, Japan). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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