SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 19: 2197-2204, 2020

Analysis of the methylation of CpG islands in the *CDO1*, *TAC1* and *CHFR* genes in pancreatic ductal cancer

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Received June 25, 2019; Accepted December 12, 2019

DOI: 10.3892/ol.2020.11340

Abstract. No difference in the gene methylation status of tumor-suppression genes between pancreatic cancer tissues and adjacent non-cancer tissues is observed. The present study investigated whether the promoter CpG islands of the cysteine dioxygenase 1 (*CDO1*), tachykinin precursor 1 (*TAC1*) and checkpoint with forkhead and ring finger domains (*CHFR*) genes were methylated in pancreatic cancer and adjacent non-cancerous pancreatic tissue in order to determine if they could be considered as markers for the detection of pancreatic cancer. A total of 38 Formalin-fixed and paraffin-embedded pancreatic adenocarcinoma tissues and their adjacent non-cancerous specimens from patients with pancreatic cancer, as well as 9 non-cancerous pancreatic samples from patients without pancreatic adenocarcinoma were obtained following surgical resection. The hypermethylation of CpG islands was detected using a methylation-specific quantitative PCR. The methylation values were calculated using the ΔCq method and were expressed as $2^{-\Delta Cq}$. The $2^{-\Delta Cq}$ value of the *CDO1* promoter from pancreatic adenocarcinoma specimens was significantly higher compared with that of adjacent non-cancerous and tumor-free pancreatic tissues (P<0.0001 and P=0.0008, respectively). The $2^{-\Delta Cq}$ value of the *TAC1* promoter of pancreatic adenocarcinoma was also significantly higher compared with that of adjacent non-cancerous tissues and tumor-free pancreatic samples (both P<0.0001). However, there was no significant difference in the $2^{-\Delta Cq}$ value of the *CHFR* promoter among the pancreatic cancer, adjacent non-cancer tissue and tumor-free pancreatic samples. Furthermore, 12 out of the 38 pancreatic adenocarcinoma cases (31.6%) presented some methylation in the *CHFR* promoter. The results from Kaplan-Meier analysis between *CHFR* promoter methylation values and the clinicopathological characteristics of patients with pancreatic adenocarcinoma demonstrated that *CHFR* promoter methylation was significantly associated with lymph node metastasis. The methylation values of *CDO1* and *TAC1* promoters in cancer tissues were higher compared with adjacent tissues. However, whether hypermethylation of *CDO1* and *TAC1* promoters may serve as a biomarker in the diagnosis of pancreatic adenocarcinoma remains unclear.

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Key words: pancreatic cancer, methylation, promoter, *CDO1*, *TAC1*, *CHFR*

Introduction

Pancreatic ductal cancer is the 7th leading cause of cancer-associated mortality worldwide (1). Although the treatment of pancreatic ductal cancer has progressed, the 5-year survival rate remains low (2-9%) (1,2). Numerous genetic alterations contribute to pancreatic cancer tumorigenesis. For example, mutation of the KRAS proto-oncogene, GTPase (*Kras*) gene is commonly observed in the early stage of pancreatic cancer (3). Furthermore, somatic mutations in the tumor protein p53 (*TP53*), SMAD family member 4 (*SMAD4*) and *p16* genes can also contribute to the progression of pancreatic cancer (3-5). In addition to genetic mutations, modifications that are not due to changes in DNA sequence, including promoter hypermethylation, are often observed in pancreatic cancer cells (6). Epigenetic silencing and transcriptional inactivation due to hypermethylation in the 5'promoter regions of specific genes, including tumor-suppressor genes, for example *hMLH1*, *BRCA1*, *p16*^{INK4a}, can contribute to cancer progression (7).

Hypermethylation of the promoter regions of the cysteine dioxygenase 1 (*CDO1*), tachykinin precursor 1 (*TAC1*) and checkpoint with forkhead and ring finger domains (*CHFR*) genes has been reported in various types of cancer (8-21), including colorectal cancer (12,15,19). The risk factors for pancreatic cancer are similar to those for colorectal cancer, and include cigarette smoking and alcohol consumption (22,23). Furthermore, patients with colorectal cancer have a significantly higher risk of developing pancreatic cancer compared

with that of the general population (24,25). The present study hypothesized therefore that pancreatic and colorectal cancer may share some genes presenting similar methylation alterations in their CG-rich region in 5'end of the promoter, called CpG islands. This alteration leads to silencing gene expression. Although DNA methylation of various genes, including *APC*, *BRCA1*, *p16*^{INK4a}, *p15*^{INK4b}, *RARβ*, and *p73,* has been examined in pancreatic cancer (26), the *CDO1, TAC1* and *CHFR* genes have not been fully described. Vedeld *et al* (12) demonstrated that the promoter region of *CDO1* in pancreatic cancer, formalin-fixed, paraffin-embedded (FFPE) samples was hypermethylated. Furthermore, Henriksen *et al* (27,28) reported that the promoter of *TAC1* in the plasmatic nucleic acids of patients with pancreatic cancer was hypermethylated, and the promoter of *CHFR* was not hypermethylated. However, the hypermethylation of these genes promoters in pancreatic cancer tissues was not compared with adjacent non-cancerous pancreatic tissues. Whether hypermethylation of these genes is already present in non-cancerous pancreatic tissues remains therefore unclear, as this was not examined by Henriksen *et al* (27,28). *CDO1*, *TAC1*, and *CHFR* methylation in pancreatic cancer tissues have not been compared with adjacent non-cancerous pancreatic tissues. The present study investigated, therefore, the methylation state of the promoter regions of the *CDO1*, *TAC1* and *CHFR* genes in pancreatic cancer and adjacent non-cancerous pancreatic tissues from patients with pancreatic cancer. In addition, it has been reported that hypermethylation of *CHFR* is associated with tumor aggressiveness in gastric and colorectal cancer (29,30). The present study hypothesized that the promoter region of these three genes may be hypermethylated, and investigated whether these genes may be considered as suitable biomarker candidates for early detection of pancreatic cancer.

Materials and methods

Patients samples. FFPE pancreatic cancer specimens [pancreatic cancer (C) group] and adjacent non-cancerous pancreatic specimens [adjacent tissue (AT) group] were obtained from 38 patients with pancreatic cancer treated at the Juntendo University Shizuoka Hospital, Japan, between January 2011 and December 2016 (Table I). Furthermore, FFPE non-cancerous pancreatic samples from 9 patients with extra-hepatic biliary tract cancers [healthy non-adjacent tissue (HN) group] were also obtained between January 2011 and December 2016 and were used as controls (Table II). In the tables, histological findings were described using the World Health Organization classification of tumors of the digestive system from 2010 (31). Clinical stages were described using the Union for International Cancer Control 8th edition classification (32).

The study protocol was performed according to the ethical guidelines of the World Medical Association and the Declaration of Helsinki, and was approved by the Ethics Committee of Juntendo University Shizuoka Hospital (approval no. 463). Patients provided consent for the use of their samples for scientific research.

Extraction and bisulfite conversion of DNA from FFPE samples. FFPE tumor and non-cancerous samples from patients with pancreatic cancer, and FFPE normal samples from patients with extra-hepatic biliary tract cancer diagnosed using hematoxylin and eosin staining sections were analyzed.

All specimens were serially cut into 10-μm thick sections. To extract DNA, sections were deparaffinized twice with xylene for 15 min and rehydrated using 100% ethanol for 3 min twice at room temperature. Proteins were digested using proteinase K (cat. no. P8107S; New England BioLabs, Inc.) dissolved in digestion lysis buffer containing denaturing agents, including sodium dodecyl sulfate, at 55˚C for 4 h. Subsequently, bisulfite conversion was performed using a Zymo EZ DNA Methylation kit (cat. no. D5002; Zymo Research Corp.) according to the manufacturer's instructions. Finally, bisulfite-modified DNA was eluted using distilled H_2O with the column from the kit. All samples were stored at -20˚C.

DNA methylation analysis. DNA methylation analysis was performed as previously described (33). The sequences of the primers (Integrated DNA Technologies, Inc.) used are presented in Table III. Following DNA bisulfite treatment, the methylation levels of the three genes *CDO1*, *TAC1* and *CHFR* was measured by quantitative methylation-specific PCR (qMSP). The qMSP levels were normalized to the values of the internal control gene β-actin. Briefly, 2 μl bisulfite-converted DNA was added to a 23-μl PCR mixture. The final reaction mixture contained 1X buffer [16.6 mM $(NH_4)_2SO_4$, 67 mM Tris pH 8.8, 6.7 mM $MgCl_2$ and 10 mM β-mercaptoethanol in nuclease-free deionized water], 200 nM sense primer, 200 nM antisense primer, 80 nM TaqMan probe (Integrated DNA Technologies, Inc.), 10 nM fluorescein reference dye (Thermo Fisher Scientific, Inc.), 0.167 mM dNTPs (Invitrogen; Thermo Fisher Scientific, Inc.) and a 1U Platinum Taq® DNA Polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). Amplification reaction of each sample was performed using MicroAmp® optical 96-well reaction plates (Applied Biosystems; Thermo Fisher Scientific, Inc.) in triplicate. The thermocycling conditions were as follows: 95˚C for 5 min, 50 cycles at 95˚C for 15 sec and 65˚C for 1 min, and 72˚C for 1 min. The StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used.

The methylation value for each sample was calculated using the ΔCq method (34) according to the following formula: $\Delta Cq=Cq_{sample}-Cq_{\beta\text{-actin}}$. A sample was considered as positively amplified when amplification was detected in ≥2 of the triplicates. For replicates that were not detected, a Cq of 100 was used, which set a minimum methylation value 0, as previously described (33). All the $Cq_{samples}$ were changed to 100 when only 1 of the 3 triplicates was amplified. The mean $2^{-\Delta Cq}$ value was calculated as follows: Methylation value=$(2^{-\Delta Cq\text{replicate }1} + 2^{-\Delta Cq\text{replicate }2} + 2^{-\Delta Cq\text{replicate }3})/3$. For a methylation value >1, a value of 1 was used, which set the maximum methylation value at 1.

Statistical analysis. The results were expressed as median values (25 and 75th percentiles). Wilcoxon signed-rank test was used to compare pancreatic cancer samples with adjacent non-cancer pancreatic samples, while Mann-Whitney U test followed by Bonferroni's correction was used to compare pancreatic cancer samples with tumor-free pancreatic samples. All clinicopathological factors were analyzed with Mann-Whitney U or Kruskal-Wallis tests. The patients' survival rates were represented using the Kaplan-Meier

Table I. Clinicopathological characteristics of the 38 patients with pancreatic ductal cancer.

Variables	Median (range) or number
Total number	38
Sex	
Male	16
Female	22
Age, years, median (range)	70 (56-82)
Tumor location	
Head	24
Body	5
Tail	9
Tumor size	
≤4 cm	24
>4 cm	14
Node involvement	
Positive	30
Negative	8
Clinical stage (UICC 8th edition)	
IB	5
IIA	3
IIB	11
III	14
IV	5
Histology (WHO classification 2010)[a]	
Wel	30
Mod	2
Por	6
Follow-up, months median (range)	14 (3-78)

[a]WHO classification 2010 corresponds to the World Health Organization for the classification of tumours of the digestive system (31). Mod, moderately differentiated carcinomas; Por, poorly differentiated ductal adenocarcinomas; Wel, well differentiated carcinomas; UICC, Union for International Cancer Control (32).

Table II. Clinicopathological characteristics of the 9 patients with extra-hepatic bile tract cancer.

Variables	Median (range) or number
Total number	9
Sex	
Male	6
Female	3
Age, years, median (range)	72 (62-79)
Tumor location	
Distal bile duct	3
Papilla of Vater	6
Node involvement	
Positive	5
Negative	7
Clinical stage (UICC 8th edition)	
IA	1
IB	3
IIB	3
IIIA	2

UICC; Union for International Cancer Control (32).

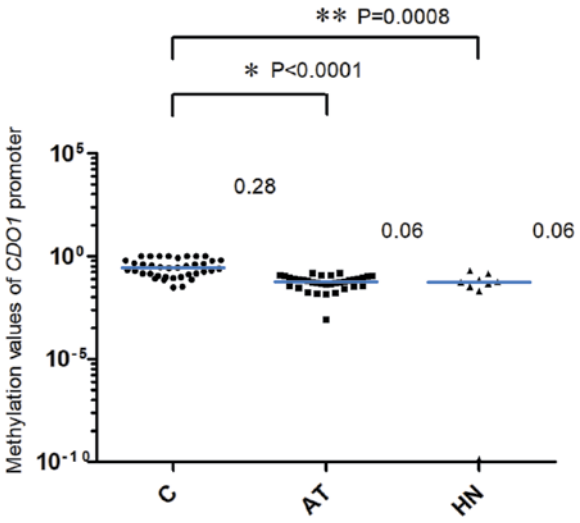

Figure 1. Methylation of the *CDO1* promoter. The $2^{-\Delta Cq}$ values of the *CDO1* promoter in the AT and HN groups were significantly lower compared with those in the C group. *P<0.0001 and **P=0.0008, AT and HN groups vs. C group, respectively. The blue horizontal lines represent median values. One single data point in the HN group was outside the axis limits. C, cancer tissues; AT, adjacent tissues; HN, the healthy non-adjacent tissue from patients with extra-hepatic biliary tract cancer; *CDO1*, cysteine dioxygenase 1.

method and were analyzed with the log-rank test for survival data. All analyses were conducted using Graph Pad Prism version 5 (GraphPad Software, Inc.) and JMP version 12.2.0 (SAS Institute, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Methylation values of the CDO1, TAC1 and CHFR promoter. The methylation values of the *CDO1* gene promoter are presented in Fig. 1. The $2^{-\Delta Cq}$ values of the *CDO1* promoter in the AT and the HN groups from patients with extra-hepatic biliary tract cancer were significantly lower compared with the those in the C group [C, 0.28 (0.13-0.64); AT, 0.06 (0.04-0.09); HN, 0.06 (0.03-0.10), median (25 and 75th percentiles); C vs. AT, P<0.0001; C vs. HN, P=0.0008]. The methylation values of the *TAC1* gene promoter are presented in Fig. 2. The $2^{-\Delta Cq}$ values of *TAC1* in the AT and HN groups were significantly lower compared with those in the C group [C, 0.13 (0.07-0.48); AT, 0.02 (0.004-0.03); HN, 0.01 (0.002-0.02), median (25 and 75th percentiles); C vs. AT, P<0.0001; C vs. HN, P<0.0001]. Conversely, the $2^{-\Delta Cq}$ values of the *CHFR* gene promoter in the C, AT and HN groups were 5.28×10^{-22} (2.82×10^{-22}-1.02×10^{-4}), 6.52×10^{-22} (2.44×10^{-22}-1.52×10^{-21}) and 2.72×10^{-22} (2.15×10^{-22}-3.78×10^{-21}), median (25 and 75th

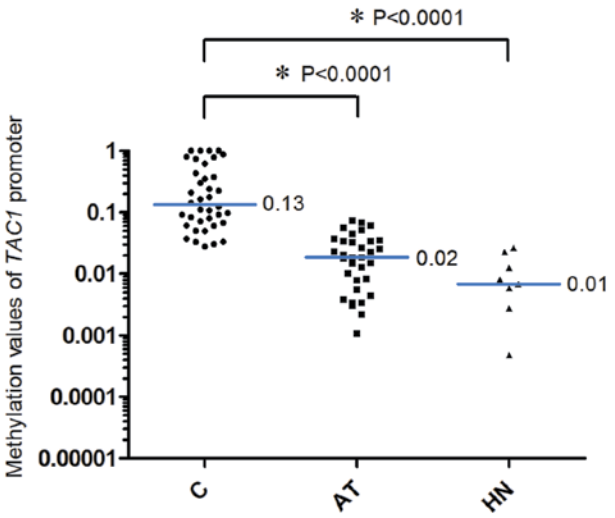

Figure 2. Methylation of the *TAC1* promoter. The $2^{-\Delta Cq}$ values of *TAC1* promoter in the AT and HN groups were significantly lower compared with those in the C group. *P<0.0001, AT and HN groups vs. C group. The blue horizontal lines represent median values. Two data points in the AT group and one data point in the HN group were outside the axis limits. C, cancer tissues; AT, adjacent tissues; HN, the healthy non-adjacent tissue from patients with extra-hepatic biliary tract cancer; *TAC1*, tachykinin precursor 1.

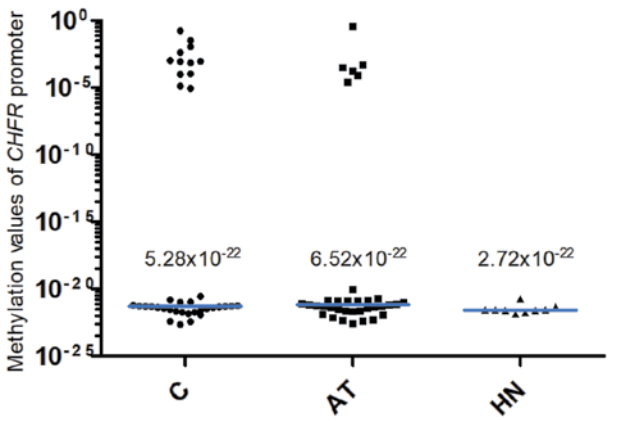

Figure 3. Methylation of the *CHFR* promoter. The $2^{-\Delta Cq}$ values of the *CHFR* gene promoter in the C, AT and HN groups were 5.28×10^{-22}, 6.52×10^{-22} and 2.72×10^{-22}, respectively. There was no significant difference in the $2^{-\Delta Cq}$ value among pancreatic cancer, adjacent non-cancer tissue and tumor-free pancreatic samples (P=0.5030, C vs. AT; P=0.1388, C vs. HN). The blue horizontal lines represent median values. C, cancer tissues; AT, adjacent tissues; HN, the healthy non-adjacent tissue from patients with extra-hepatic biliary tract cancer; *CHFR*, checkpoint with forkhead and ring finger domains.

percentiles), respectively (Fig. 3). When comparing the $2^{-\Delta Cq}$ values of the *CHFR* promoter among pancreatic cancer specimens, no significant difference was observed among pancreatic cancer, adjacent non-cancer tissue and tumor-free pancreatic samples (Fig. 3). In addition, 12 of the 38 cases in the C group (31.6%) exhibited methylation values of the *CHFR* gene promoter $>1.0 \times 10^{-6}$.

Association between the patients' clinicopathological characteristics and the methylation values. The association between the patients' clinicopathological characteristics and the $2^{-\Delta Cq}$ values of the *CDO1*, *TAC1* and *CHFR* promoter regions in the cancer tissues was investigated (Table IV). No significant association was observed between the $2^{-\Delta Cq}$ values of the three gene promoters and the clinicopathological variables tumor stage, tumor size or tumor differentiation. However, a significant association between the $2^{-\Delta Cq}$ values of the *CHFR* promoter and node metastasis was observed. The $2^{-\Delta Cq}$ values of the *CHFR* promoter in node metastasis-positive cases were significantly higher compared with those in node metastasis-negative cases (P=0.0484).

The association between the $2^{-\Delta Cq}$ values of the *CDO1*, *TAC1* and *CHFR* genes in pancreatic cancer tissues and the overall survival rates of patients was determined using Kaplan-Meier analysis (Figs. 4-6). The values from 5 cases were excluded because these patients had stage IV cancer and underwent palliative resection. The cut-off values were defined as the median of the *CDO1* and *TAC1* promoter $2^{-\Delta Cq}$ values, as previously described (13), and were 0.25 and 0.11 for the *CDO1* and *TAC1* genes, respectively. The cut-off value for the *CHFR* $2^{-\Delta Cq}$ value was 1.0×10^{-6}. The results demonstrated that there was no significant association between the $2^{-\Delta Cq}$ values of the *CDO1*, *TAC1* and *CHFR* genes and the overall survival rates of patients with pancreatic cancer [P=0.1709 (Fig. 4), P=0.2683 (Fig. 5) and P=0.6985 (Fig. 6), respectively].

Discussion

The epigenetic hypermethylation of the promoter CpG islands of tumor-suppressor genes, including *APC, BRCA1, p16*^{INK4a} can induce transcription inactivation during tumorigenesis, which is often observed in pancreatic cancer (6). Previous studies in pancreatic cancer reported frequent genetic abnormalities in *Kras* gene activation, but also in the epigenetic inactivation of *p16*^{INK4a}, *p53* and *SMAD4* in >50% of pancreatic ductal cancer cases (4,5). Guo *et al* (26) demonstrated that the promoters of the genes APC regulator of WNT signaling pathway, BRCA1 DNA repair associated, *p16*^{INK4a}, *p15*^{INK4b}, retinoid acid receptor-β and p73 were hypermethylated in patients with pancreatic ductal cancer. However, the promoter hypermethylation of *TAC1* and *CHFR* remains unclear. Hypermethylation of the *CDO1* gene promoter in only 20 pancreatic cancer tissues has been evaluated by Vedeld *et al* (12), who reported that promoter of *CDO1* in 18 of the 20 pancreatic cancer tissues using FFPE samples is hypermethylated. However, the association between *CDO1* gene promoter methylation status and clinicopathological characteristics of patients was not analyzed.

CDO1 is a protein that catalyzes the conversion of cysteine to cysteine sulfinic acid, which helps decreasing the levels of reactive oxygen species (ROS) in the cell (35). Furthermore, depletion of *CDO1* increases oxidative stress in tumor cells, which induces tumor cell resistance to ROS and metastasis (9). Hypermethylation of the *CDO1* CpG island promoter has been reported in various types of cancer, including breast (9,13), lung (non-small cell type) (14), colon (12), kidney (clear cell type) (11), esophageal (10) and pancreatic cancer (12). Vedeld *et al* (12) reported that *CDO1* silencing occurs in early-stage tumorigenesis of colorectal cancer and that *CDO1* hypermethylation is detected in normal colorectal mucosa samples. These results suggest that genetic methylation could occur prior to detection of any histological, anatomical or morphological changes. The results from the present study demonstrated that the $2^{-\Delta Cq}$ values of the *CDO1* promoter regions in the adjacent

Table III. Primer sequences for quantitative methylation-specific PCR.

Gene	Forward, 5'-3'	Reverse, 5'-3'	Probe 5'	Product size, bp	Annealing temperature, °C
CDO1	CGTTTTTTTCGTTTTATTTTCGTCG	CCTCCGACCCTTTTTATCTACG	TGTGGTTCGCGACGTTGGGACGT	69	65
TAC1	TCGGGTTATTTCGTTTCGTATTTGTTC	CACTATCCCTCGCCGCAACG	AGGTGGTCGCGTTGGGGGCGTCGT	69	65
CHFR	TTAGAGGTTTTTGCGTTTCGCG	CGACTCCGCTTTAACTACCG	TTGGTTGGCGGCGGCGTTTATTAAGAGCG	70	65
β-actin	TAGGGAGTATATAGGTTGGGGAAGTT	AACACACAATAACAAACACAA ATTCAC	TGTGGGGTGGTGATGGAGGAGGTTTAG	103	65

CDO1, cysteine dioxygenase 1; *CHFR*, checkpoint with forkhead and ring finger domains; *TAC1*, tachykinin precursor 1.

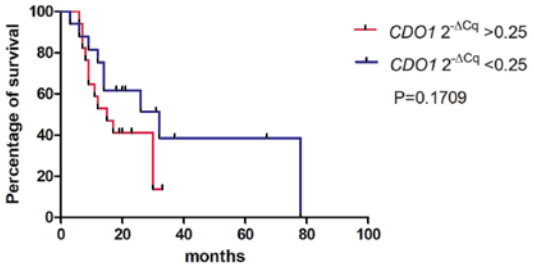

Figure 4. Survival rates for high and low *CDO1* promoter $2^{-\Delta Cq}$ values estimated by the Kaplan-Meier method. There was no significant difference between the high and low hypermethylation groups of the *CDO1* promoter. The cut-off value was defined as 0.25, which was the median $2^{-\Delta Cq}$ value of the *CDO1* promoter. In total, 5 patients with stage IV pancreatic cancer who underwent palliative resection were excluded. *CDO1*, cysteine dioxygenase 1.

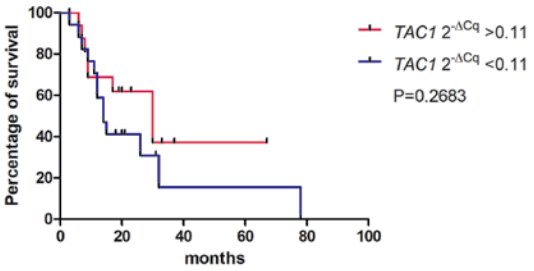

Figure 5. Survival rates for high and low *TAC1* promoter $2^{-\Delta Cq}$ values estimated by the Kaplan-Meier method. There was no significant difference between the high and low hypermethylation groups of the *TAC1* promoter. The cut-off value was defined as 0.11, which was the median $2^{-\Delta Cq}$ value of the *TAC1* promoter. In total, 5 patients with stage IV pancreatic cancer who underwent palliative resection were excluded. *TAC1*, tachykinin precursor 1.

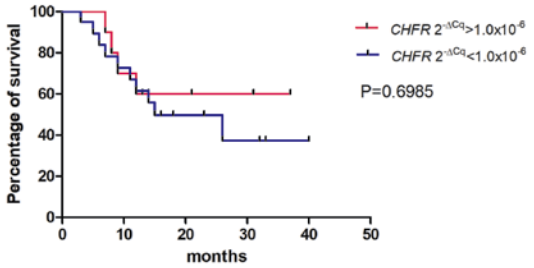

Figure 6. Survival rates for high and low *CHFR* promoter $2^{-\Delta Cq}$ values estimated by the Kaplan-Meier method. There was no significant difference between the high and low hypermethylation groups of the *CHFR* promoter. The cut-off value was defined as 1.0×10^{-6}, which was considered the positively hypermethylated. $2^{-\Delta Cq}$ value of the *CHFR* promoter. In total, 5 patients with stage IV pancreatic cancer who underwent palliative resection were excluded. *CHFR*, checkpoint with forkhead and ring finger domains.

non-cancerous pancreatic tissues of patients with pancreatic cancer were lower compared with those of patients with pancreatic cancer tissues; however, methylation did occur in these histologically normal-appearing tissues. These results also suggested that *CDO1* methylation may occur before detection of morphological changes in pancreatic cancer. The reason why the methylation value of *CDO1* promoter was elevated in HN group remains unclear. *CDO1* promoter

Table IV. Comparison between the patients' clinicopathological characteristics and the methylation values of *CDO1*, *CHFR* and *TAC1*.

Variable	*CDO1* $2^{-\Delta Cq}$ value, median (25 and 75th percentiles)	*TAC1* $2^{-\Delta Cq}$ value, median (25 and 75th percentiles)	*CHFR* $2^{-\Delta Cq}$ value, median (25 and 75th percentiles)
Node metastasis			
Positive	0.23 (0.11-0.69)	0.13 (0.06-0.65)	1.01×10^{-21} (4.05×10^{-22}-3.97×10^{-4})
Negative	0.42 (0.22-0.64)	0.15 (0.08-0.28)	3.23×10^{-22} (6.56×10^{-22}-5.37×10^{-22})
P-value	0.3151	0.9857	0.0484[a]
Tumor size, cm			
≤4	0.33 (0.11-0.63)	0.09 (0.06-0.40)	6.17×10^{-22} (3.5×10^{-22}-5.42×10^{-4})
>4	0.24 (0.16-0.82)	0.21 (0.10-0.76)	4.75×10^{-22} (1.65×10^{-22}-1.32×10^{-21})
P-value	0.7345	0.1805	0.2587
Differentiation			
Wel, mod	0.23 (0.12-0.57)	0.12 (0.07-0.42)	5.87×10^{-22} (3.48×10^{-22}-6.87×10^{-4})
Por	0.91 (0.30-1.00)	0.46 (0.04-0.84)	3.37×10^{-22} (1.47×10^{-22}-8.70×10^{-22})
P-value	0.0680	0.5750	0.1223
Stage			
IB	0.39 (0.23-0.73)	0.09 (0.06-0.24)	3.56×10^{-22} (1.63×10^{-22}-5.34×10^{-4})
IIA	0.61 (0.18-0.65)	0.22 (0.12-0.80)	1.47×10^{-22} (3.86×10^{-23}-3.87×10^{-22})
IIB	0.21 (0.08-0.40)	0.14 (0.06-0.43)	6.46×10^{-22} (4.62×10^{-22}-9.23×10^{-4})
III	0.22 (0.11-0.87)	0.10 (0.04-0.58)	2.83×10^{-21} (3.46×10^{-22}-3.97×10^{-4})
IV	0.44 (0.14-1.01)	0.62 (0.12-0.83)	5.22×10^{-22} (2.66×10^{-22}-5.70×10^{-3})
P-value	0.6009	0.5566	0.2562

[a]P<0.05. Comparison between the three gene values and node metastasis, tumor size and differentiation was analyzed with Mann-Whitney U test. Comparison between the three gene values and tumor stage was analyzed with Kruskal-Wallis test. *CDO1*, cysteine dioxygenase 1; *CHFR*, checkpoint with forkhead and ring finger domains; *TAC1*, tachykinin precursor 1; Wel, well differentiated adenocarcinomas; Mod, moderately differentiated carcinomas; Por, poorly differentiated ductal adenocarcinomas.

hypermethylation in pancreatic cancer tumorigenesis appears therefore to be similar to that in colorectal cancer.

TAC1 encodes preprotachykinin-1, which is converted to neurokinin A or substance P (36). Since neurokinin A inhibits cell proliferation in normal cell (37), *TAC1* is therefore considered a tumor-suppressor gene, and hypermethylation of the *TAC1* CpG island promoter has been observed in various types of cancer, including lung (non-small cell type) cancer (14), colon cancer (15), head and neck cancer (16), uterus cancer (17) and pancreatic cancer (27,28). Patai *et al* (38) reported that *TAC1* promoter is hypermethylated in the precancerous condition of colorectal sessile serrated adenomas. Subsequently, *TAC1* gene methylation is likely to occur during the early stage of tumorigenesis in colorectal cancer (38). In the present study, *TAC1* promoter methylation was higher in pancreatic cancer tissues compared with that in adjacent non-cancerous tissues. Similar to *CDO1*, hypermethylation of *TAC1* promoter was also detected in adjacent non-cancerous tissues, suggesting that *TAC1* promoter methylation may occur during the early stage of tumorigenesis in pancreatic cancer.

CHFR encodes a protein that regulates DNA synthesis and delays entry into mitosis during the G2 phase (39). Hypermethylation of the *CHFR* gene is crucial during esophageal and gastric cancer tumorigenesis (20,21,40). *CHFR* promoter methylation could also provide clinical information, including clinical response to taxane chemotherapy, since patients with gastric or esophageal cancer and with *CHFR* hypermethylation, or with *CHFR* gene silencing in gastric and esophageal cancer are thought to have good clinical responses to docetaxel and paclitaxel treatments (20,41). Pelosof *et al* (18) suggested therefore that docetaxel should be used for the treatment of patients with colorectal cancer who presented with *CHFR* promoter methylation. Cleven *et al* (19) reported that hypermethylation of *CHFR* in patients with colorectal cancer indicates poor prognosis of stage ll colorectal cancer. Subsequently, *CHFR* methylation may serve for selecting chemotherapy agents for cancers of the digestive tract system, and could be considered a putative prognostic indicator in cancer therapy. The results from the present study demonstrated that *CHFR* promoter hypermethylation only occurred in 12 out of 38 cases (31.6%) and did not predict pancreatic cancer tumorigenesis. Since the response rate to gemcitabine and nab-paclitaxel is 23% in the MPACT trial (42), the present study hypothesized that 31.6% as a *CHFR* hypermethylation frequency might be reasonable. The present study also demonstrated that patients with lymph node metastasis had higher $2^{-\Delta Cq}$ values of *CHFR* gene promoter methylation compared with those of patients without lymph node metastasis. In gastric and colorectal cancer, *CHFR* methylation has been reported to be associated with lymph node metastasis and prognosis (29,30). Although the present study did not report the prognostic value of *CHFR* gene promoter methylation in

patients with pancreatic cancer, it demonstrated that *CHFR* gene methylation was associated with lymph node metastasis in patients with pancreatic cancer. However, two populations presenting highly different *CHFR* methylation values in the C and AT groups were observed. These observations may be caused by cell contamination, such as tumor cells migration to non-tumor tissue, although absence of cancer was confirmed by histopathological analysis. However, the *CHFR* methylation levels were increased in the cancer-free pancreas or precancerous condition, which has been previously described (27).

This study presented some limitations. Firstly, the sample size was small. Secondly, evaluation of the methylation status of the three genes in normal pancreatic tissue or tissues from patients with chronic pancreatitis. Thirdly, the association between disease recurrence of patients treated with chemotherapy, in particular paclitaxel, and their overall survival rate was not assessed. In addition, further investigation on the role of *CHFR* as a prognostic and predictive marker is required.

Pancreatic cancer is characterized by virulent tumor and a low 5-year survival rate (6%) mainly because it is frequently diagnosed at a late stage (1,2). The present study demonstrated that *CDO1* and *TAC1* promoter methylation values were similar in all stages. These results suggest that the hypermethylation of *CDO1* and *TAC1* promoters may be related to early events in pancreatic cancer.

The methylation values of *CDO1* and *TAC1* promoters in cancer tissues were higher compared with adjacent tissues. However, whether the hypermethylation of *CDO1* and *TAC1* may serve as biomarkers for the diagnosis of pancreatic cancer remains unknown. The role of CHFR promoter methylation in pancreatic cancer remains unclear and requires further investigation.

Acknowledgements

The authors would like to thank Professor Ryo Wada (Department of Pathology, Juntendo University Shizuoka Hospital) for his histopathological diagnosis, and Ms. Junko Kawai (Department of Pathology, Juntendo University Shizuoka Hospital) for preparing FFPE sections.

Funding

This study was supported by a Grant-in-Aid from MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2015-2019 (grant no. S1511008L) and the Banks Family Foundation. Tomoaki Ito was supported by the TORAY Company.

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

HM, KS, TI and MB designed this study. HO, MS and TK collected FFPE samples and clinical information. TI and AH performed the experiments. HM and TI analyzed the data and drafted the manuscript. MB revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Ethical approval and consent to participate

The study protocol followed the ethical guidelines of the World Medical Association and the Declaration of Helsinki, and was approved by the Ethical Committee of Juntendo University Shizuoka hospital. Patients provided informed consent for the use of their samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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