DNA hypermethylation of nonspecific cytotoxic cell receptor protein 1 and poor prognosis of pancreatic cancer

MAI NAKAMURA[1-3], TERUKI HAGIWARA[2], KAZUHIKO YAMADA[1,3], TORU IGARI[4], YUKI FUKUMURA[5], AKIO SAIURA[6], NOBUYUKI TAKEMURA[1], NORIHIRO KOKUDO[1] and YUKI I. KAWAMURA[2,3]

[1]Department of Surgery, National Center for Global Health and Medicine, Japan Institute for Health Security, Tokyo 162-8655, Japan; [2]Clinical Research Advancement Section, National Institute of Global Health and Medicine, Japan Institute for Health Security, Tokyo 162-8655, Japan; [3]Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; [4]Pathology Division of Clinical Laboratory, National Center for Global Health and Medicine, Tokyo 162-8655, Japan; [5]Department of Human Pathology, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; [6]Department of Hepatobiliary Pancreatic Surgery, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan

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Abstract. Pancreatic cancer (PC) is a highly fatal malignancy and one of the leading causes of cancer-related deaths globally. Pancreatitis and inflammation-induced epigenetic changes, such as DNA methylation, have been reported to be involved in carcinogenesis and progression of PC. The present study examined the precise expression and DNA methylation status of nonspecific cytotoxic cell receptor protein 1 (NCCRP1), identified as a methylation target gene in esophageal cancers, in paired adjacent normal pancreas and PC tissues. NCCRP1 expression was immunohistochemically analyzed using formalin-fixed, paraffin-embedded sections of 73 patients with PC who underwent surgery. The DNA methylation status was analyzed in 52 paired adjacent normal and PC tissues using pyrosequencing. In normal pancreatic tissues, NCCRP1 expression was restricted to acinar cells and absent in ductal and islet cells. Most PCs (64/73) showed a loss of NCCRP1 expression, whereas NCCRP1 expression was observed in 9 cases. Among the NCCRP1-positive cases, 8 (89%) were classified as anaplastic carcinoma, an undifferentiated subtype of PC. When human PC cell lines were treated with 5-aza-2'-deoxycytidine, a DNA-methyltransferase inhibitor, NCCRP1 transcription was induced. Pyrosequencing analysis revealed that the promoter regions of NCCRP1 were highly DNA methylated in PC tissues compared with paired adjacent normal tissues. Furthermore, PC cases with DNA hypermethylated (>18.88%) NCCRP1 exhibited significantly poorer survival than those with relatively low levels of NCCRP1 methylation (P=0.0164). The data collectively suggested that NCCRP1 expression was frequently lost in PC, likely due to promoter hypermethylation, resulting in poor prognosis. This indicates the potential of NCCRP1 as a diagnostic and prognostic biomarker and a therapeutic target for patients with PC.

Correspondence to: Dr Yuki I. Kawamura, Clinical Research Advancement Section, National Institute of Global Health and Medicine, Japan Institute for Health Security, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan
E-mail: kawamura.y@jihs.go.jp

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Introduction

Pancreatic cancer (PC) is a lethal malignancy with a 5-year survival rate of approximately 10% in the USA (1). It is the third leading cause of cancer death in the US (2), and fourth in Japan (3). Radical resection remains the only way to cure this malignancy, despite advancements in systemic chemotherapy and molecular targeted therapy. One reason for the poor prognosis of PC is that most patients present no symptoms until the advanced stage, losing the chance for curative surgery at initial diagnosis (1). Additionally, the specific origin of each pancreatic tumor is still not fully understood. Recent studies using mouse models have demonstrated that pancreatic damage prompts acinar cells to acquire ductal features, eventually contributing to tumorigenesis (4). Epigenetic mechanisms induced by cellular injury or inflammation in the pancreas are involved in the acinar-to-ductal metaplasia (ADM) process, transforming acinar cells into ductal-like cells with plasticity (5), which plays a crucial role in PC development.

The epigenetic landscape of PC is actively explored (6). For example, DNA methylation, one of the epigenetic mechanisms, has been found to be upregulated in chronic pancreatitis (7), a well-known major inducer of ADM (8). Consequently, genes whose expression is perturbed by aberrant DNA methylation may play a critical role in the ADM process. Tobacco use, alcohol consumption, chronic pancreatitis, obesity, and diabetes are well-known modifiable risk factors for PC (9,10) that induce epigenetic abnormalities. Numerous studies have investigated the relationship between these risk factors and epigenetic mechanisms (11), including DNA methylation,

histone modifications, chromatin remodeling, and non-coding RNA-mediated gene regulation. These mechanisms regulate gene expression without altering the underlying DNA sequence. Considering this knowledge, developing an understanding of the molecular biology, mechanisms, and origins of pancreatic tumors will be a primary challenge for clinical oncologists.

We previously identified 56 genes with decreased expression due to DNA hypermethylation in esophageal cancer compared with normal esophageal mucosa tissues (12). Among these gene, we focused on the NCCRP1 gene because Zuo *et al* (13) reported that NCCRP1 was expressed in normal pancreas but decreased in PC. However, the precise expression pattern of NCCRP1 protein in pancreatic tissue, specifically in acinar and ductal cells, has not been identified. Additionally, the mechanism regulating NCCRP1 expression in PC has not been elucidated. In this study, we examined the precise expression and DNA methylation status of NCCRP1, which has been reported as one of the methylation target genes in esophageal cancers, in paired adjacent normal pancreas and PC tissues. We found that NCCRP1 expression is frequently lost in PC, probably due to promoter hypermethylation. We further analyzed the relationship between DNA methylation status of NCCRP1 and PC prognosis and found that hypermethylation of the *NCCRP1* in PC may predict a poorer prognosis in PC patients.

Materials and methods

Patients. We enrolled 63 patients diagnosed with PC who underwent surgery at the National Center for Global Health and Medicine (NCGM) between January 2011 and March 2022. To analyze the association with anaplastic carcinoma of the pancreas (ACP), we additionally enrolled 3 ACP cases resected at NCGM before 2010 and 9 ACP cases resected at Juntendo University between January 2011 and September 2018. In all 75 PC cases, 73 formalin-fixed, paraffin-embedded sections were used for IHC to evaluate NCCRP1 expression. Consent was retrospectively obtained from these patients in an opt-out format by posting documents on the website for a comprehensive study in accordance with the guidelines of the National Center for Global Health and Medicine Research Ethics Committee (approval no: 2417). For this study, we accessed the medical records of the patients from April 2024 to October 2025 and retrospectively obtained patient data. Clinical and pathological tumor stages were evaluated using the Union for International Cancer Control (UICC) TNM Classification of Malignant Tumors, 8th edition (14). In addition, 52 of 75 PC cases with available frozen samples were examined by pyrosequencing to assess DNA methylation status of NCCRP1. Written informed consent was obtained from these 52 patients before sample collection (approval no: 2464).

Surgical procedure and treatment. We perform standard pancreatectomy for PC by experienced pancreatic surgeons at our institution. Distal pancreatectomy is performed if the cancer is located to the body and tail of the pancreas, with or with no concomitant splenectomy. Pancreaticoduodenectomy (Whipple procedure) is performed to remove the head of the pancreas involving the resection of the distal stomach, common bile duct, duodenum, gallbladder. Pancreaticoduodenectomy with portal vein (PV) and/or superior mesenteric vein (SMV) resection is performed for PC when PV/SMV involvement is determined before or during surgery. Patients who are in a good physical condition after surgery receive adjuvant chemotherapy with S-1 (TS-1; Taiho Pharmaceutical) for 6 months, based on the JASPAC 01 study (15). In recent years, neoadjuvant chemotherapy (NAC) has emerged as a preferred choice for patients with resectable, borderline resectable, and locally advanced PC, compared to upfront surgery, and is considered for patients with a predicted R0 resection. We have currently been using two cycles of the GS regimen (gemcitabine plus S-1) as NAC, based on the results of the Prep-02/JSAP05 study (16).

Immunohistochemical analysis. Immunohistochemical (IHC) staining was performed in accordance with the method previously reported by Yamada *et al* (17) An anti-NCCRP1 antibody (HPA048141; Sigma-Aldrich, St. Louis, MO) and the ImmPACTTM DAB Peroxidase Substrate kit (Vector Laboratories, Burlingame, CA) were used to determine NCCRP1 immunoreactivity. All slides were reviewed by two observers without access to clinical or pathological data. Based on the proportion of NCCRP1-positive areas in the immunohistochemically stained tumor tissue, patients were classified as those who were NCCRP1-negative (≤30% NCCRP1-positive tumor cells) and NCCRP1-positive groups (>30% NCCRP1-positive tumor cells).

Cell lines and culture. Human cell lines derived from PC (Capan-2, AsPC-1, and MiaPaca-2), those derived from head and neck squamous cell carcinoma (FaDu and Detroit562), and those derived from lung cancers (HTB-174 and HCC827) were obtained from the American Type Culture Collection. A human colorectal cancer cell line HCT116 and an esophageal squamous cell carcinoma (ESCC) cell line KYSE140 were obtained from the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT. Capan-2 and HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal calf serum (FCS). AsPC-1, HTB-174, and HCC827 cells were maintained in RPMI1640 medium supplemented with 10% FCS. MiaPaca-2 cells were cultured in DMEM medium supplemented with 10% FCS and 1 mM sodium pyruvate. FaDu and Detroit562 cells were cultured in Minimum Essential Medium supplemented with 10% FCS and 1-mM sodium pyruvate. KYSE140 were maintained in Ham's F12/RPMI1640 medium containing 2% FCS. HCT116 cells with genetic disruption of DNMT1 (DNMT1 KO) or DNMT3b (DNMT3b KO), which were previously established and molecularly validated (18), were used in the present study. In some experiments, the cells were cultured in a 24-well plate at a density of $5x10^4$ cells/well for 18 h, and then treated with 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich) or butyrate (Selleck Chemicals, Houston, TX, USA) for 4 days. These human cell lines, which were authenticated by the supplier using short tandem repeat testing, were passaged in our laboratory for fewer than 6 months after resuscitation.

NCCRP1 ectopic expression. To construct the expression vector of the *NCCRP1* gene, a DNA fragment encoding the full-length ORF (OriGene Technologies, Rockville, MD, USA)

was used as a template, and the *NCCRP1* gene was amplified with polymerase chain reaction (PCR) using Primestar (Takara Bio, Shiga, Japan). The *NCCRP1* fragment was ligated into the pIRES2-EGFP vector (CLONTECH, Palo Alto, CA, USA) using an In-Fusion HD Cloning Kit (CLONTECH). The resulting vector or vector alone was transfected into MiaPaca-2 cells using lipofectamine LTX reagent (Life Technologies, Carlsbad, CA, USA). Stably transfectants were isolated using a MoFlo™ XDP Cell Sorter (Beckman Coulter, Brea, CA, USA).

Cell proliferation assay. MiaPaca-2 transfectants were seeded in 96-well plates at 5,000 cells/well, and cell proliferation was measured using an MTT assay kit (Nacalai Tesque, Kyoto, Japan). Cell proliferation was investigated at 24-h intervals.

Reverse transcription-quantitative PCR. Total RNA was isolated from cultured cells using the RNA easy Mini Kit (QIAGEN, Hilden, Germany). After treating RNA with DNase I, double-stranded complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using ABI TaqMan. Threshold cycle numbers (Ct) were determined using the Sequence Detector software and transformed using the $2^{-\Delta\Delta Cq}$ method (19) as described by the manufacturer, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the calibrator gene according to the manufacturer's instructions. The TaqMan Gene expression assay IDs for the genes used in this study were NCCRP1, Hs01583969_m1 and GAPDH, Hs00266705_g1 (Applied Biosystems).

Pyrosequencing. Bisulfite modification was performed by using an EpiTect Bisulfite Kit (QIAGEN). To assess the NCCRP1 methylation status, pyrosequencing was performed with PyroMark Gold Q24 reagents and a PyroMark Q24 pyrosequencing machine (QIAGEN). The PCR primers used in this study were 5'-GGATAGAGAAGGAAGTGGTAGAG-3' and 5'-TTAAATCCCCAAACTTCCTACCC-3'. The primer used for pyrosequencing was 5'-ATGTTGGATTTTAATGAGG-3'.

Methylation-specific PCR (MSP). Using a PyroMark PCR Kit (QIAGEN) as previously reported, MSP was performed to assess the ZNF382 methylation status (20). The methylation-specific primers were 5'-GGCGATTAACGGGTCGTTTC-3' and 5'-AAAATTTCCAAACCCGACTCG-3'. The unmethylated primers were 5'-GTGGTGATTAATGGGTTGTTTT-3' and 5'-CAAAATTTCCAAACCCAACTCA-3'. Following agarose gel electrophoresis of the PCR products, each band was quantified using the NIH ImageJ software, and the ZNF382 methylation status was presented as the methylated-to-unmethylated ZNF382 ratio.

Statistical analysis. Data were expressed as means ± standard deviation. One-way ANOVA followed by Tukey's multiple comparisons test was used to analyze three or more groups. Paired Student's t test was used for the comparison of DNA methylation frequencies between paired adjacent normal and PC tissues. Clinicopathological characteristics were compared between groups using the unpaired Student's t-test for continuous variables (age), whereas the Chi-square test or Fisher's exact test, as appropriate, was used for categorical variables (sex, tumor type, pT status, pN status, pM status, and disease stage). These analyses were applied to comparisons between NCCRP1-positive and NCCRP1-negative patients, as well as between NCCRP1 hypermethylation and non-hypermethylation groups. Comparisons between *NCCRP1*-transfected and mock-transfected cells were performed using the unpaired Welch's t-test. Overall survival (OS) was defined as the time from the date of surgery to death from any cause or the last known follow-up visit. Comparisons between groups were performed using the log-rank test. Statistical analyses were performed using the Prism 7 statistical program (GraphPad Software, Inc., La Jolla, CA, USA). In the univariate and multivariate analyses, the Cox proportional hazards regression model was used to evaluate the risk ratio using JMP statistical analysis software (version 17; SAS Institute Inc., Cary, NC, USA). All tests were two-tailed, and P-values of <0.05 were considered statistically significant.

Results

Participants. We enrolled 66 patients diagnosed with PC who underwent surgery at the National Center for Global Health and Medicine (NCGM) and 9 patients diagnosed with ACP who underwent surgery at Juntendo University. Among 75 PC cases, we performed IHC on 73 cases to investigate the protein expression and localization of NCCRP1 in paired adjacent normal and PC tissues, and bisulfite-pyrosequencing for 52 cases to assess the frequency of NCCRP1 methylation in PC tissues. There was no significant difference in mean age between the IHC group (70.1 years) and the pyrosequencing group (71.0 years). Eleven (15.1%) cases for IHC analysis and 16 (30.8%) cases for pyrosequencing received NAC. The IHC group of 73 cases included 46 patients (63%) received pancreaticoduodenectomy (PD), and 27 patients (37%) received distal pancreatectomy (DP). The pyrosequencing group of 52 cases included 32 (61.5%) PD and 20 (38.5%) DP patients. The characteristics of the cases in each assay are summarized in Table I.

NCCRP1 expression in adjacent normal and cancerous tissues of pancreas. In adjacent normal pancreatic tissues obtained from patients with PC, NCCRP1 protein was expressed in acinar cells but not in ductal and islet cells (Fig. 1A). The protein expression of NCCRP1 was confined to the cytoplasm, with no detection in the nuclei of acinar cells. In contrast, we found the loss of NCCRP1 expression in the majority of pancreatic carcinomas (64/73, 86.3%) (Fig. 1B). To investigate the clinicopathological features of NCCRP1-expressing PC, we classified PC cases into the NCCRP1-negative and NCCRP1-positive groups. No significant differences in clinical features including sex, pT stage, pN stage, pM stage, or cancer stage, were observed between these groups. Of the nine NCCRP1-positive cases, eight were pathologically classified as anaplastic carcinoma (ACP), which is characterized by pleomorphic giant and spindle-shaped cells lacking glandular differentiation (Fig. 1C). The ratio of ACP in NCCRP1-positive group was significantly higher than that of pancreatic ductal adenocarcinoma (PDAC), which exhibits typical ductal structures with desmoplastic stroma ($P<0.0001$) (Table II).

Table I. Characteristics of the patients with pancreatic cancer included in each assay.

Characteristics	Immunohistology (n=73)	Pyrosequencing (n=52)
Mean age ± SD, years	70.1±10.8	71.0±9.5
Sex, n (%)		
Male	47 (64.4)	33 (63.5)
Female	26 (35.6)	19 (36.5)
pT classification, n (%)		
T1/2	11 (15.1)	11 (21.2)
T3/4	62 (84.9)	41 (78.8)
pN classification, n (%)		
N0	26 (35.6)	19 (36.5)
N1	47 (64.4)	33 (63.5)
pM classification, n (%)		
M0	70 (95.9)	48 (92.3)
M1	3 (4.1)	4 (7.7)
Cancer stage[a], n (%)		
IA/IB/IIA	26 (35.6)	19 (36.5)
IIB/III/IV	47 (64.4)	33 (63.5)
Tumor site, n (%)		
Head	46 (63.0)	32 (61.5)
Tail	27 (37.0)	20 (38.5)
Neoadjuvant chemotherapy, n (%)	11 (15.1)	16 (30.8)

[a]Based on the Union for International Cancer Control TNM Classification of Malignant Tumors, 8th edition (14).

Aberrant DNA hypermethylation contributes the loss of NCCRP1 in PC. To clarify whether DNA methylation in the NCCRP1 promoter contributes to the reduced NCCRP1 expression in PCs, we compared its mRNA expression levels in three PC cell lines to those in cells treated with 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor. In all cell lines, treatment with 5-aza-dC resulted in increased expression of NCCRP1 mRNA (Fig. 2A). Next, we quantified NCCRP1 methylation in PC tissues by analyzing CpG methylation levels in 52 paired adjacent normal and PC tissue samples. Significantly elevated NCCRP1 methylation was observed in cancer tissues compared to paired adjacent normal mucosa (P<0.0001, Fig. 2B), suggesting that NCCRP1 expression was silenced by DNA hypermethylation in PCs. To identify which DNA methyltransferase contributes to NCCRP1 promoter methylation, we examined NCCRP1 expression in human colorectal cancer HCT116 cells with knockout (KO) of DNMT1 or DNMT3b. NCCRP1 expression was higher in DNMT1 KO cells than in parental HCT116 cells, whereas DNMT3b KO cells showed no change (Fig. 2C), suggesting that DNMT1 is primarily responsible for NCCRP1 promoter methylation. Furthermore, we evaluated the effect of 5-aza-2'-deoxycytidine in other cancer cell types, including head and neck squamous cell carcinoma, esophageal cancer, and lung cancer. Treatment with 5-aza-2'-deoxycytidine induced NCCRP1 expression in all cancer cell types examined (Fig. 2D), indicating that DNA methylation-mediated NCCRP1 silencing occurs across multiple cancer types.

To explore alternative regulatory mechanisms, we treated PC cell lines with butyrate, a histone deacetylase inhibitor.

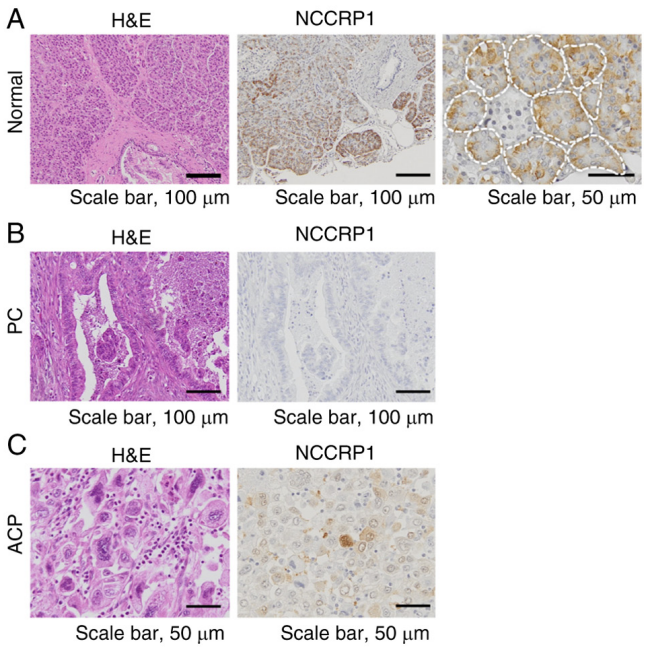

Figure 1. Representative images of formalin-fixed, paraffin-embedded adjacent normal pancreatic tissues and PC specimens stained with H&E or anti-NCCRP1 antibody. (A) NCCRP1 was expressed in acinar cells of normal pancreatic tissues. Representative acinar structures are indicated by white dotted outlines. (B) NCCRP1 expression was decreased in PC. (C) NCCRP1 expression was detected in ACP. ACP, anaplastic carcinoma of the pancreas; NCCRP1, nonspecific cytotoxic cell receptor protein 1; PC, pancreatic cancer.

Table II. Cancer types of NCCRP1-negative and -positive patients with pancreatic cancer.

Characteristics	Total	NCCRP1-negative	NCCRP1-positive	P-value
No. of patients (%)	73	64 (87.7)	9 (12.3)	
Cancer subtype, n				
Ductal adenocarcinoma	52	51	1	
Well differentiated	16	16	0	
Moderately differentiated	23	23	0	
Poorly differentiated	13	12	1	
Adenosquamous carcinoma	2	2	0	
Mucinous carcinoma	4	4	0	
Anaplastic carcinoma	13	5	8	<0.0001[a]
Acinar cell carcinoma	2	2	0	

[a]Statistically significant (P<0.005). The ratio of NCCRP1-positive cases was compared with that in ductal adenocarcinoma using Fisher's exact test. A P-value was calculated for anaplastic carcinoma only. Statistical comparisons were not performed for other cancer subtypes due to small sample sizes and the presence of 0 counts. NCCRP1, nonspecific cytotoxic cell receptor protein 1.

Butyrate increased NCCRP1 mRNA expression in all three cell lines, suggesting that histone modifications, in addition to DNA methylation, may also contribute to NCCRP1 downregulation (Fig. 2E).

Correlation between survival and NCCRP1 expression or methylation status in PC. To analyze the relation between the methylation status of the *NCCRP1* gene and clinicopathological features of PC, we classified PC cases into low and high methylation groups based on a cut-off value (18.88%) determined using receiver operating characteristic (ROC) curve analysis (Fig. 3A). Out of the 52 cases examined, 34 (65.4%) demonstrated hypermethylation of NCCRP1. No statistically significant associations were observed in patients' characteristics or tumor stages between these groups (Table III). Kaplan-Meier estimates were then evaluated within both NCCRP1 expression and NCCRP1 methylation groups. In the prognostic analyses of patients assessed by IHC, there was no difference between NCCRP1-positive and NCCRP1-negative groups when considering the entire cohort (Fig. 3B). To clarify the function of the *NCCRP1*, we prepared *NCCRP1*-transfected and mock-transfected MIAPaCa cells and investigated the effect of *NCCRP1* expression on their growth. Stably *NCCRP1*-transfected MIAPaCa cells displayed >30,000-fold higher *NCCRP1* expression than mock-transfected counterparts (Fig. 3C); however, *NCCRP1*-transfected and mock-transfected cells demonstrated no significant difference in growth (Fig. 3D). In contrast, among patients analyzed by pyrosequencing, those with hypermethylated NCCRP1 exhibited significantly poorer survival compared to those with low-methylated NCCRP1 (Fig. 3E, P=0.0164). In the same cohort, we investigated the methylation status of ZNF382, one of methylation makers already examined in PC (20), and classified PC cases into low and high methylation groups on the basis of the cut-off value (0.289) determined by ROC curve analysis (Fig. 3F). Patients with ZNF382 hypermethylation also exhibited significantly poorer survival (Fig. 3G, P=0.0025). Univariate analysis of OS revealed that NCCRP1 hypermethylation (P=0.014), advanced pT classification (P=0.022), advanced pN classification (P=0.012), advanced pStage (P=0.013), and lack of adjuvant chemotherapy (P=0.003) were significant risk factors (Table IV). No significant differences were observed with respect to sex or age; however, poorer survival was noted in the hypermethylated NCCRP1 group compared with the low-methylated group among male and younger patients (<75 years; Fig. 3H). In addition, poorer survival in the hypermethylated group was observed only among patients who received adjuvant chemotherapy (Fig. 3H). Multivariate analysis identified NCCRP1 hypermethylation [hazard ratio (HR), 2.602; 95% confidence interval (CI), 1.012-6.689; P=0.0471], advanced stage (HR, 2.977; 95% CI, 1.215-7.294; P=0.0017), and lack of adjuvant chemotherapy (HR, 3.771; 95% CI, 1.487-9.563; P=0.005) as independent prognostic factors (Table V).

Discussion

In the present study, we first demonstrated that NCCRP1 is silenced by aberrant DNA hypermethylation in PC. Furthermore, we clearly indicated significance of the DNA methylation status of the acinar cell-specific gene, *NCCRP1*, as a prognostic factor. Our findings also indicate that NCCRP1 methylation status is a better indicator for selecting patients with poor prognosis than its protein expression detected by antibody.

We found that the patients with PC in which the *NCCRP1* gene was DNA hypermethylated have poorer prognosis as compared with the patients with lower methylation levels of the *NCCRP1* gene. To our knowledge, there are no reports of *NCCRP1* being methylated in cancer, including PC, with prognostic value. Our immunostaining results showed that almost all cases had no NCCRP1 expression, suggesting that evaluation of NCCRP1 expression by IHC is not suitable to predict prognosis. Instead, DNA methylation status of the *NCCRP1* is a better indicator for selecting patients with poor prognosis. There are several reports identifying DNA methylation correlated with survival prognosis in PDAC (21-23). Among these abnormally methylated genes in PDAC, ZNF154 and ZNF382

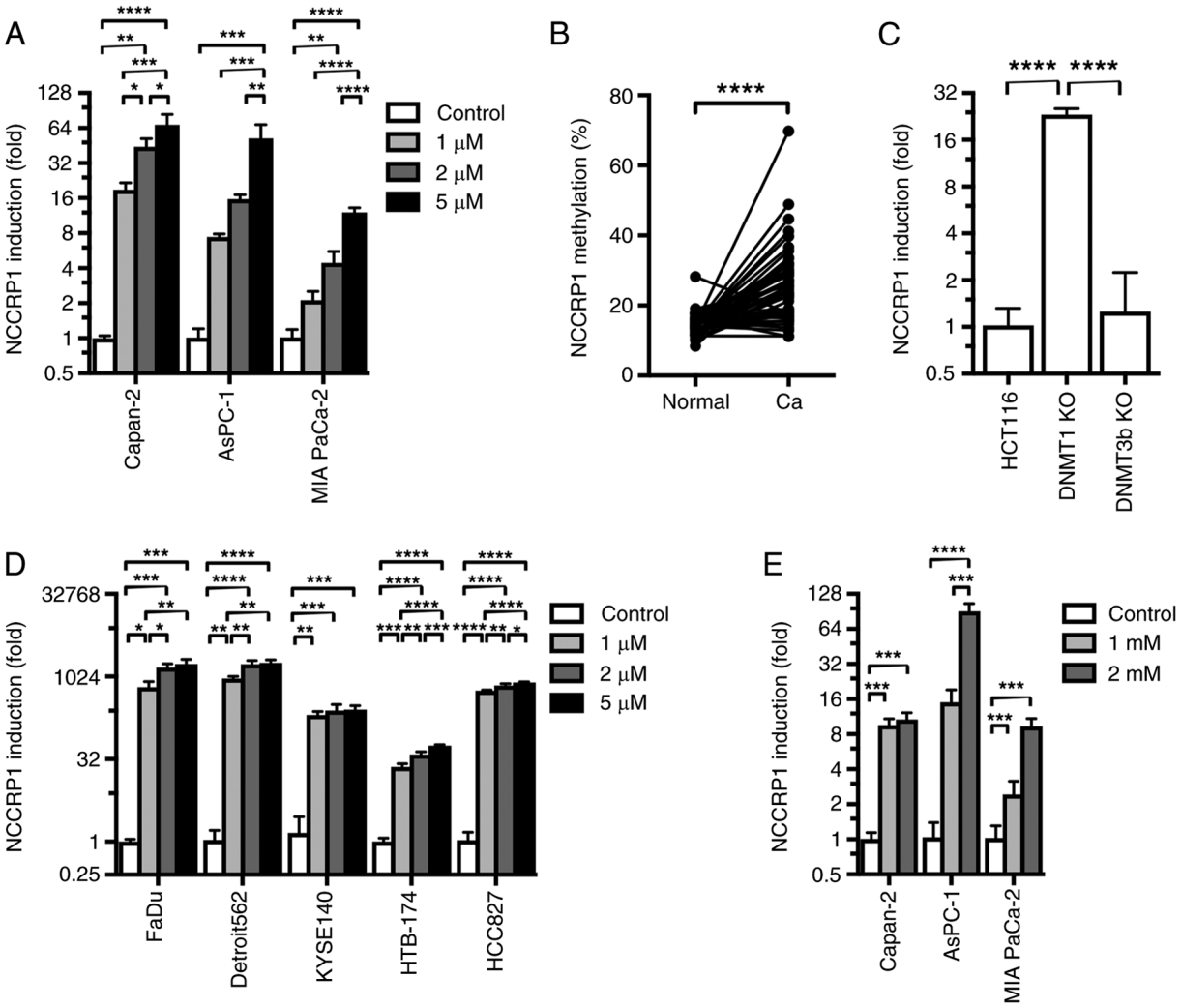

Figure 2. NCCRP1 expression is silenced by DNA hypermethylation in PC. (A) NCCRP1 mRNA induction, which is defined as the fold increase in NCCRP1 mRNA expression levels in PC cell lines treated with 1, 2 or 5 μM 5-aza-2'-deoxycytidine relative to untreated control cells. Data from one representative experiment out of three independent experiments are presented as the mean ± SD of assays conducted in triplicate, because the absolute values varied substantially between experiments; however, similar trends were observed across all independent experiments. (B) DNA methylation of NCCRP1 was determined via pyrosequencing of paired samples from 40 patients with PC. The data indicated hypermethylation in cancerous tissues compared with the mean levels in paired adjacent normal tissues. (C) Induction of NCCRP1 mRNA in DNMT1 KO and DNMT3b KO cells compared with parental HCT116 cells. Data from one representative experiment out of three are presented as the mean ± SD of assays conducted in triplicate, because substantial inter-experiment variability in absolute values precluded pooling of data across independent experiments; however, consistent trends were reproducibly observed in all experiments. (D) Induction of NCCRP1 mRNA in head and neck squamous cell carcinoma cell lines (FaDu and Detroit562), an esophageal cancer cell line (KYSE140), and lung cancer cell lines (HTB-174 and HCC827) following treatment with 1, 2 or 5 μM 5-aza-2'-deoxycytidine presented as fold increases in induction relative to untreated cells. Data from one representative experiment out of three are presented as the mean ± SD of assays conducted in triplicate, because substantial inter-experiment variability in absolute values precluded pooling of data across independent experiments; however, consistent trends were reproducibly observed in all experiments. (E) Induction of NCCRP1 mRNA in PC cell lines following treatment with 1 or 2 mM butyrate presented as fold increases in induction relative to untreated controls. Data from one representative experiment out of three are presented as the mean ± SD of triplicate assays, because substantial inter-experiment variability in absolute values precluded pooling of data across independent experiments; however, consistent trends were reproducibly observed in all experiments. (A and C-E) Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (B) Statistical analysis was performed using a paired Student's t-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Ca, cancerous tissue; DNMT, DNA methyltransferase; KO, knockout; NCCRP1, nonspecific cytotoxic cell receptor protein 1; PC, pancreatic cancer.

function as tumor suppressors, which may contribute to lower survival rates in PC (22,24). Therefore, loss of NCCRP1 by aberrant DNA methylation may has a significant role in PC progression. Moreover, NCCRP1 and ZNF382 had comparable proportions of high methylation cases (65.4% vs. 61.5%), with concordance in most cases (both high methylation in 55.8%; both low in 28.8%). A minority (9.6%) demonstrated high NCCRP1 but low ZNF382 methylation, suggesting that NCCRP1 can detect additional high-risk cases missed by existing markers. Ongoing clinical trials of epigenetic drugs in solid tumors (25) may encourage combination strategies involving demethylating agents (e.g., 5-aza analogs) with chemotherapy for PC. Although adjuvant chemotherapy is a standard treatment after resection of PC, around half of resected patients can complete adjuvant chemotherapy and over 75% of patients experience a recurrence within the first two years post-resection (26-28). Methylation status information will be valuable in selecting patients who are suitable for personalized treatments. Wu *et al* (7) reported that circulating cell-free DNA (cfDNA) methylation signatures can

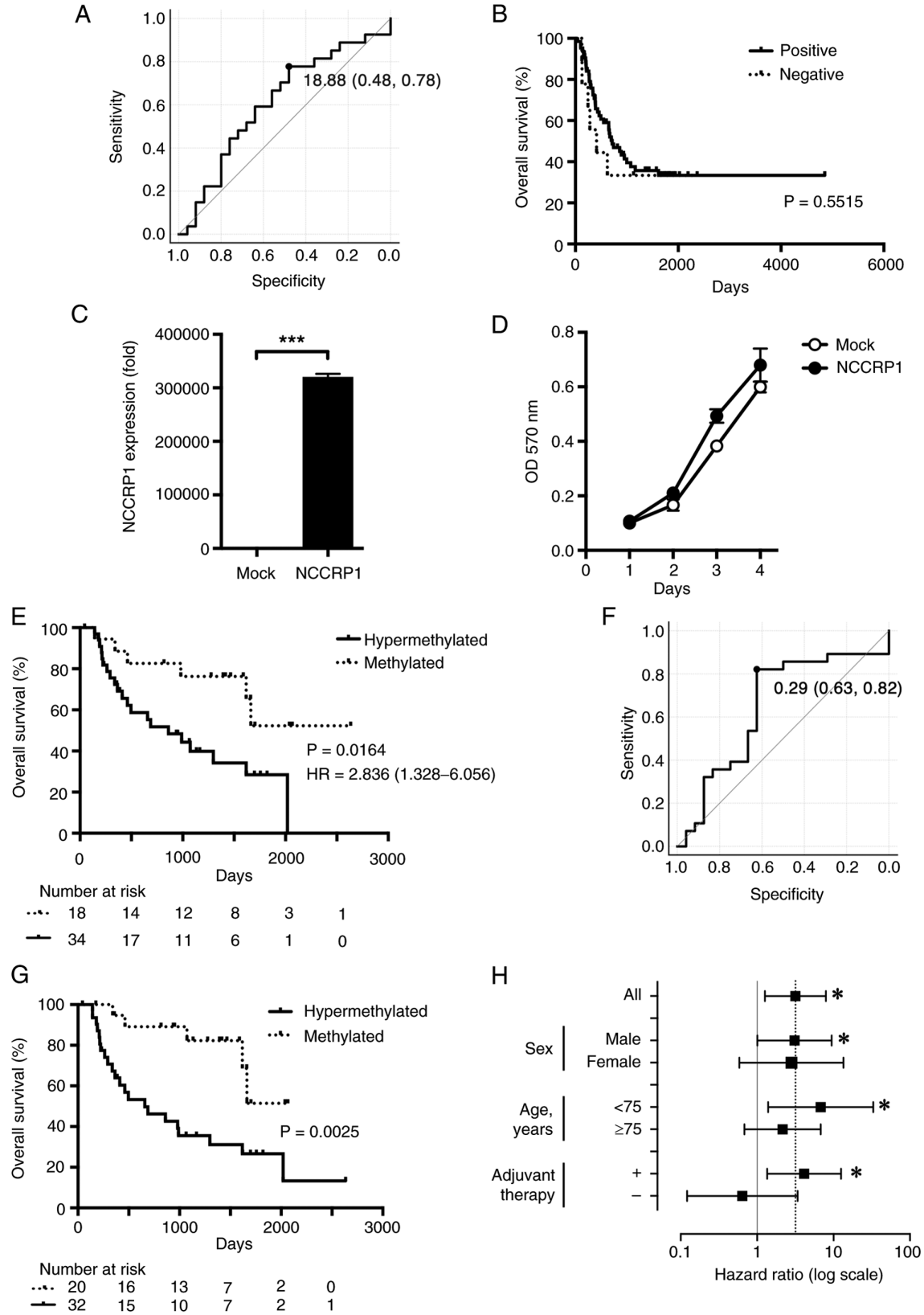

Figure 3. Kaplan-Meier curves of overall survival in patients with PC. Hypermethylated NCCRP1 in PC is correlated with poor prognosis. (A) ROC curve analysis to determine the optimal cutoff value for NCCRP1 hypermethylation. (B) Overall survival rates of NCCRP1-positive and -negative patients exhibited no significant difference. (C) *NCCRP1* mRNA levels in mock-transfected and *NCCRP1*-transfected MIAPaCa cells. (D) Growth curves of *NCCRP1*-transfected and mock-transfected MIAPaCa cells as determined using an MTT assay. (E) Patients with hypermethylated NCCRP1 exhibited a significantly poorer overall survival. Numbers at risk are provided below the Kaplan-Meier curves. (F) ROC curve analysis to determine the optimal cutoff value for ZNF382 hypermethylation. (G) Patients with hypermethylated ZNF382 had a significantly poorer overall survival. (H) Forest plot showing subgroup analyses. (B, E and G) Overall survival was analyzed using the Kaplan-Meier method, and differences between groups were assessed using the log-rank test. (A and F) ROC curve analyses were performed. (C) Comparisons were performed using the unpaired Welch's t-test. (D) Comparisons between NCCRP1-transfected and mock-transfected cells at each individual timepoint were performed using the unpaired Welch's t-test. *P<0.05 and ***P<0.001. HR, hazard ratio; NCCRP1, nonspecific cytotoxic cell receptor protein 1; OD, optical density; PC, pancreatic cancer; ROC, receiver operating characteristic; ZNF382, zinc finger protein 382.

Table III. Nonspecific cytotoxic cell receptor protein 1 methylation status and clinical features of pancreatic cancer.

Characteristics	Hypermethylation	Methylation	P-value
No. of patients (%)	34 (65.4)	18 (34.6)	
Mean age ± SD, years	70.9±9.9	71.3±9.2	0.886
Sex, n (%)			0.727
Male	21 (61.8)	12 (66.7)	
Female	13 (38.2)	6 (33.3)	
pT classification, n (%)			0.482
T1/2	6 (17.7)	5 (27.8)	
T3/4	28 (82.4)	13 (72.2)	
pN classification, n (%)			0.389
N0	11 (32.4)	8 (44.4)	
N1	23 (67.7)	10 (55.6)	
pM classification, n (%)			0.999
M0	31 (91.2)	17 (94.4)	
M1	3 (8.8)	1 (5.6)	
Cancer stage[a], n (%)			0.389
IA/IB/IIA	11 (32.4)	8 (44.4)	
IIB/III/IV	23 (67.6)	10 (55.6)	
Tumor site, n (%)			0.963
Head	21 (61.8)	11 (61.1)	
Tail	13 (38.2)	7 (38.9)	
Neoadjuvant chemotherapy, n (%)	9 (26.5)	7 (38.9)	0.356
Adjuvant chemotherapy, n (%)	20 (58.8)	15 (83.3)	0.073

[a]Based on the Union for International Cancer Control TNM Classification of Malignant Tumors, 8th edition (14). Continuous variables were compared using the unpaired Student's t-test. Categorical variables were compared using the χ^2 test or Fisher's exact test, as appropriate.

distinguish PC from chronic pancreatitis. Additionally, liquid biopsies containing tumor-derived cfDNA could be obtained before surgical resection. Although further studies are needed to develop cfDNA-based assays for clinical application and to assess the feasibility of detecting NCCRP1 methylation in cfDNA as a noninvasive prognostic biomarker, preoperative assessment of NCCRP1 methylation using liquid biopsies may provide additional clinical value in decision-making for neoadjuvant therapy.

NCCRP1 was initially identified in fish with a cytolytic function (29). Therefore, the role of NCCRP1 in humans is mostly unknown. At the molecular level, NCCRP1 functions as a paralog within the F-box superfamily of proteins, which are key components of E3 ubiquitin ligase complexes known for their regulatory roles in the cell cycle (30). Zuo *et al* (13) reported a relationship between ubiquitination-related genes, including NCCRP1, and PC prognosis. Although further studies using mass spectrometry to identify proteins that coprecipitated with NCCRP1 are warranted, it may play a similar regulatory role in PC. To examine the functional significance of NCCRP1 loss in PC, we generated NCCRP1-transfected and mock-transfected MIAPaCa cells. However, no significant difference in growth was observed between the two groups. In this study, we also demonstrated that NCCRP1 protein expression was undetectable in almost all PC tissue samples, irrespective of methylation status. These findings collectively suggest that NCCRP1 molecular functions may not have a major influence on PC prognosis. Existing research, for instance, Zhou *et al* (31) demonstrated that elevated NCCRP1 expression correlates with poor prognosis in triple-negative breast cancer, a subtype known for its high malignancy and poorer outcomes. Similarly, in PC, Zuo *et al* (13) reported that despite the down-regulation of NCCRP1 expression in tumor tissue, patients with high NCCRP1 expression have a poorer prognosis, which aligns with our study results in which ACP with poor prognosis showed significantly higher expression of NCCRP1. On the other hand, Miwa *et al* (32) observed in ESCC that low NCCRP1 expression was associated with poor prognosis, emphasizing that NCCRP1 function is context-dependent.

In adjacent normal pancreas obtained from patients with PC, NCCRP1 expression is restricted to acinar cells. Although further studies using lineage-tracing mouse models are warranted, the observation of NCCRP1 hypermethylation in PC may indicate that the origin of PC could be acinar cells, where NCCRP1 is silenced by aberrant DNA methylation induced by risk factors such as pancreatitis, smoking, alcohol consumption, or diabetes. The understanding of the cellular origins of pancreatic tumors remains elusive. Previous studies have shown that both acinar and ductal cells possess the capability

Table IV. Univariate analysis of the risk factors for overall survival in 52 patients with pancreatic cancer.

Variables	Hazard ratio	95% CI	P-value
NCCRP1			
Hypermethylated	3.171	1.266-7.944	0.014[a]
Methylated	1.000	-	
Age, years			
≥75	1.385	0.643-2.984	0.406
<75	1.000	-	
Sex			
Male	1.000	-	
Female	1.038	0.478-2.255	0.925
pT classification			
T3/4	4.230	1.237-14.461	0.022[a]
T1/2	1.000	-	
pN classification			
≥N1	3.038	1.276-7.234	0.012[a]
N0	1.000	-	
pM classification			
M1	2.387	0.552-10.320	0.244
M0	1.000	-	
Cancer stage[b]			
IIB, III, IV	3.005	1.263-7.147	0.013[a]
IA, IB, IIA	1.000	-	
Tumor site			
Head	1.303	0.593-2.866	0.510
Tail	1.000	-	
Neoadjuvant chemotherapy			
Yes	1.323	0.601-2.913	0.487
No	1.000	-	
Adjuvant chemotherapy			
Yes	1.000	-	
No	3.562	1.537-8.258	0.003[a]

Univariate analyses were performed using the Cox proportional hazards regression model. [a]Statistically significant (P<0.05). [b]Based on the Union for International Cancer Control TNM Classification of Malignant Tumors, 8th edition (14). NCCRP1, nonspecific cytotoxic cell receptor protein 1.

Table V. Multivariate analysis of the risk factors for overall survival in 52 patients with pancreatic cancer.

Variables	Hazard ratio	95% CI	P-value
NCCRP1			
Hypermethylated	2.602	1.012-6.689	0.047[a]
Methylated	1.000	-	
Age, years			
≥75	1.315	0.560-3.092	0.530
<75	1.000	-	
Sex			
Male	1.000	-	
Female	1.378	0.573-3.312	0.474
Cancer stage[b]			
IIB, III, IV	2.977	1.215-7.294	0.017[a]
IA, IB, IIA	1.000	-	
Adjuvant chemotherapy			
Yes	1.000		
No	3.771	1.487-9.563	0.005[a]

Multivariate analyses were performed using the Cox proportional hazards regression model. [a]Statistically significant (P<0.05). [b]Based on the Union for International Cancer Control TNM Classification of Malignant Tumors, 8th edition (14). NCCRP1, nonspecific cytotoxic cell receptor protein 1.

to form PDAC (33). Recently, Del Poggetto *et al* (4) reported that acinar cells are the origin of cancer in mouse models. Our findings indicate that PC may have originated from distinct cell types: those with NCCRP1 hypermethylation (likely derived from acinar cells) and those with NCCRP1 low-methylation (likely derived from ductal cells). This indicates that cases where NCCRP1 was low-methylated may have developed into cancer through alternative mechanisms. It is also possible that both acinar cells and ductal cells are originated from common stem cells (34). Beer *et al* (35) summarized the existence of progenitor cells exhibiting squamous epithelial characteristics among pancreatic ductal, islet, and acinar cells. In zebrafish and murine models, these progenitor cells were demonstrated to possess the ability to differentiate into both ductal and acinar cells, categorized as centroacinar cells. Since there are no reports indicating that prognosis differs depending on cell origin, further studies are needed.

ACP has significantly higher expression of NCCRP1. According to the World Health Organization (WHO) classification of pancreatic tumors, ACP is defined as undifferentiated carcinoma in which a significant component of the neoplasm has no specific signs of differentiation (36). Although prognosis of ACP is thought to be poor compared to other types of PC, the clinical features and treatment of ACP remain unknown because of its rarity. A stratified analysis of ACP cases showed a trend toward poorer survival among NCCRP1-positive patients (HR=3.37), although this was not statistically significant (P=0.1651, data not shown). Interpretations regarding NCCRP1 expression patterns in ACP should be made cautiously due to the limited sample size. Nonetheless, ACP may undergo carcinogenesis through a different molecular pathway, including DNA methylation status.

A limitation of the present study is the use of a small cohort for pyrosequencing. In addition, several limitations are considered, including a small sample size and its retrospective nature, potentially leading to biases. This study also lacked analysis of patients with late-stage PC because these patients are often not candidates for surgical resection. The omission of the lineage-tracing mouse models is also a limitation of the

present study. Although the limited number of samples in the methylation-survival analysis reduces statistical power, this study is the first to demonstrate that NCCRP1 hypermethylation in PC correlates with poor survival prognosis. The poorer survival in patients with hypermethylated NCCRP1 suggests the importance of epigenetic landscapes in determining patient prognosis and proposes a novel therapeutic target.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YIK conceived and designed the study. MN, TH and YIK acquired data. MN and YIK analyzed and interpreted data. MN, KY, TI, YF, AS, NT and NK contributed to the collection of clinical specimens and analyzed clinicopathological data. MN and YIK drafted the manuscript. MN, KY, TH and YIK obtained funding. MN and YIK confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the National Center for Global Health and Medicine Research Ethics Committee (approval no. 2464; Shinjuku, Tokyo, Japan), and written informed consent was obtained from 52 patients before sample collection and subsequent pyrosequencing. For the remaining patients in whom archived pathological specimens were used only in the immunohistochemical analysis, the requirement for written informed consent was waived because the study was retrospective and non-interventional. Instead, an opt-out document was posted on the hospital website before the study commenced and consent was retrospectively obtained in accordance with the guidelines of the National Center for Global Health and Medicine Research Ethics Committee (approval no. 2417).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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