ary fluids using a DNeasy Tissue Kit. Extracted DNA was quantified, and 500 ng of DNA was modified with sodium bisulfite using a Methylamp™ DNA modification kit.

Bisulfite-pyrosequencing

Bisulfite-pyrosequencing analysis was performed as described previously[17,24]. Briefly, polymerase chain reaction (PCR) was run in a 25 μL volume containing 50 ng bisulfite-treated DNA, 1× MSP buffer, 1.25 mmol/L dNTP, 0.4 μmol/L of each primer and 0.5 U of Jump-Start REDTaq DNA Polymerase. The PCR protocol for bisulfite sequencing entailed 5 min at 95 ℃; 40 cycles of 1 min at 95 ℃, 1 min at 60 ℃ and 1 min at 72 ℃; and a 7 min final extension at 72 ℃. The biotinylated PCR product was purified, made single-stranded and used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The PCR products were bound to Streptavidin Sepharose beads HP; then, the beads containing the immobilized PCR product were purified, washed and denatured using a 0.2 mol/L NaOH solution. After adding 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was performed using a PSQ96MA system and Pyro Q-CpG software. Primer sequences for LINE-1 methylation were as previously described[25].

MethyLight assay

The MethyLight assay was performed as previously described[25,26]. Based on previous studies and our preliminary results, we analyzed 10 promoter CpG island loci: aryl-hydrocarbon receptor repressor, adenomatous polyposis coli, calcium channel, voltage dependent, T type α1G subunit, insulin-like growth factor 2, O-6-methylguanine-DNA methyltransferase, neurogenin 1, CDKN2A, runt-related transcription factor 3 (RUNX3), secreted frizzled-related protein 1, and ubiquitin carboxyl-terminal esterase L1 (UCHL1). β-actin was used as the internal reference gene to quantify modified DNA levels in the samples[26]. Primers, probes and the percentage of methylated reference (PMR, *i.e.*, the degree of methylation) were as previously described[27-29]. We used a PMR cutoff of 4 to distinguish methylation-positive (PMR > 4) from methylation-negative (PMR ≤ 4) samples based on previously validated data[29].

5-AZA-2'-deoxycytidine and/or Trichostatin A treatment

To examine the role of CpG methylation and histone deacetylation in the silencing of UCHL1, cancer cells were treated with 2 or 5 mol/L 5-AZA-2'-deoxycytidine (5-AZA-dC) (Sigma) for 72 h or 100 nmol/L Trichostatin A (TSA) for 24 h. The cells were also treated with 2 μmol/L 5-AZA-dC for 72 h, followed by 100 nmol/L TSA for an additional 24 h. The timing and sequencing of 5-AZA-dC and/or TSA were based on similar preliminary studies, as well as published studies[30]. After the treatment, UCHL1 expression was analyzed by real-time RT-PCR.

Real-time quantitative PCR

Total RNA from cell lines was extracted using an extraction kit. cDNA was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase with random hexamers. qRT-PCR was performed using the TaqMan real-time PCR system as previously described[31]. A comparative threshold cycle (Cт) was used to determine the gene expression relative to the control (calibrator). Control reactions were performed without reverse transcriptase.

Statistical analysis

Mean methylation levels of LINE-1 were compared using *t* tests, the Welch test, or one-way ANOVA with a post hoc Games-Howell test. LINE-1 methylation levels and hypermethylation of tumor-associated genes were assessed for associations with clinicopathological parameters using *t* tests, the Welch test, the χ^2 two-tailed test, Fisher's exact test, the Mann-Whitney test, or one-way ANOVA. A P value < 0.05 was considered statistically significant. A P value between 0.05 and 0.10 was considered to indicate a trend toward an association.

RESULTS

Hypomethylation of LINE-1 in pancreatic and biliary fluids from patients with pancreatobiliary cancers

We performed bisulfite pyrosequencing to quantitatively analyze LINE-1 promoter methylation as a surrogate for genome-wide methylation (Figure 1). The mean level of LINE-1 methylation in pancreatic fluids was slightly but significantly lower in patients with pancreatic cancer than in those with noncancerous pancreatic disease (Figure 2). The mean level of LINE-1 methylation in biliary fluids was significantly lower in patients with pancreatobiliary cancer than in those with noncancerous pancreatobiliary disease. There was no correlation between LINE-1 methylation levels and clinicopathological characteristics in patients with pancreatic or pancreatobiliary cancers. The mean level of LINE-1 methylation in pancreatic cancer tissues was significantly lower than that in pancreatic fluids from the corresponding patients.

Analysis of CpG island hypermethylation of tumor-related genes

We next assessed the methylation levels of CpG islands of well-characterized tumor-suppressor and tumor-associated genes. Using MethyLight assays, we analyzed 10 genes. CpG island hypermethylation of tumor-associated genes was detected at various frequencies. The results are summarized on the basis of each individual marker (Tables 1 and 2). Methylation of several genes, such as UCHL1 and RUNX3, was cancer-specific (Figure 3). Some cancer samples showed methylation in many genes, suggesting that these cancers have a CpG island hypermethylator phenotype. We failed to find any significant correlation between the methylation of these genes and clinicopathological features or between CpG island meth-

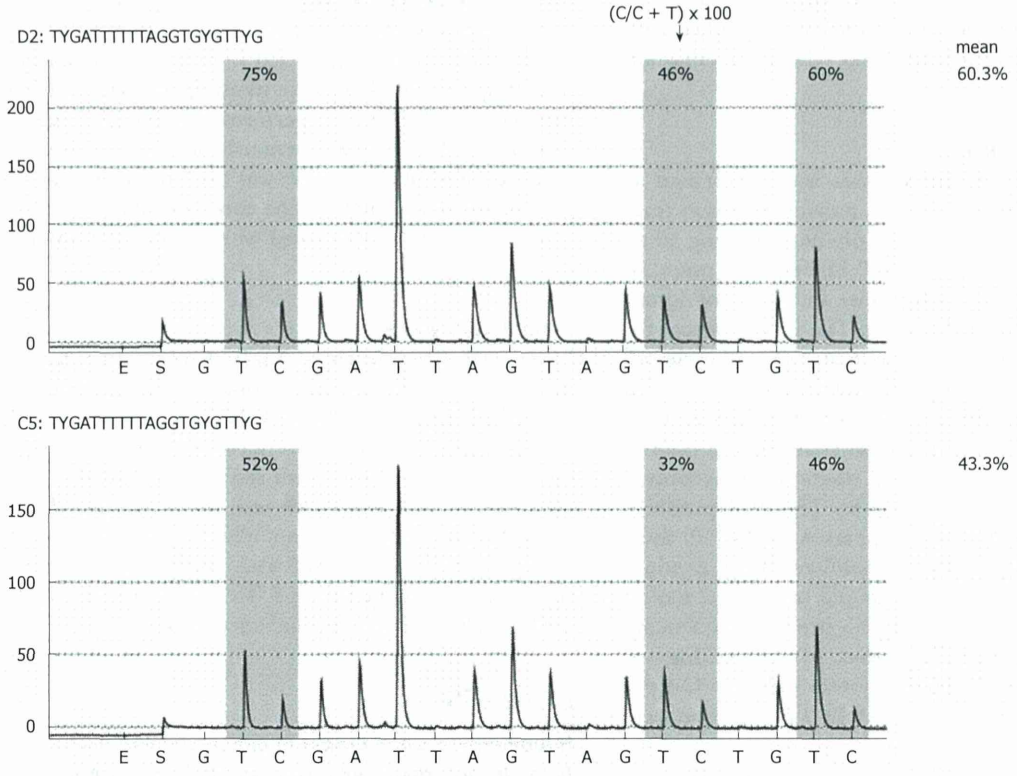

Figure 1 Representative long interspersed nuclear element-1 methylation analysis by pyrosequencing. Long interspersed nuclear element-1 methylation analysis in biliary fluids from patients with noncancerous pancreatic disease (chronic pancreatitis, upper panel) and pancreatic cancer (lower panel) is shown.

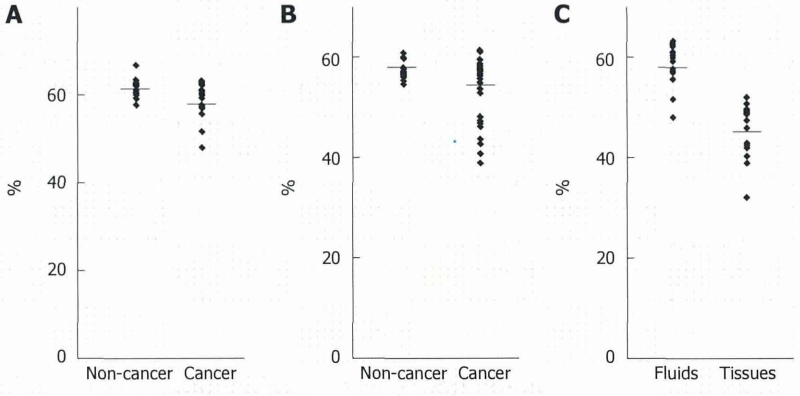

Figure 2 Analysis of long interspersed nuclear element-1 methylation levels using pancreatobiliary fluids. A: Comparison of the long interspersed nuclear element-1 (LINE-1) methylation levels in pancreatic fluids between pancreatic cancer and noncancerous pancreatic disease; B: Comparison of the LINE-1 methylation levels in biliary fluids between pancreatobiliary cancer and noncancerous pancreatobiliary disease; C: Comparison of the LINE-1 methylation levels in pancreatic cancer between pancreatic fluids and tissues.

ylation and LINE-1 methylation levels.

Among the cancer-specific hypermethylated genes in pancreatic fluids, the *UCHL1* gene was most frequently (67%) detected in pancreatic cancer and served as the most useful single marker for the detection of pancreatic cancer (Figure 3A). Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic fluids was the most useful combined marker for the detection of pancreatic cancer.

Among the cancer-specific hypermethylated genes in biliary fluids, the *UCHL1* gene was most frequently (70%) detected in pancreatobiliary cancer and served as the most useful single marker for the detection of pancreatobiliary cancer (Figure 3C). Hypermethylation of the *UCHL1* and *RUNX3* genes in biliary fluids was the most useful combined marker for detection of pancreatobiliary cancer.

The pancreatic and biliary fluids obtained from pan-

Table 1 CpG islands hypermethylation of tumor-associated genes in pancreatic fluids

	Age (yr)	Sex	Stage	*UCHL1*	*RUNX3*	*CDKN2A*	*IGF2*	*CACNA1G*	*AHRR*	*SFRP1*	*MGMT*	*APC*	*NEUROG1*
NC1	68	M		MN	MN	MN	MN	MN	MN	MP	MP	MN	MN
NC2	65	M		MN	MN	MN	MN	MP	MN	MN	MN	MN	MN
NC3	59	M		MN	MN	MP	MN	MN	MN	MN	MN	MN	MP
NC4	70	F		MN	MN	MN	MP	MN	MN	MN	MN	MN	MN
NC5	43	F		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC6	55	M		MN	MN	MN	MN	MP	MN	MN	MN	MN	MN
NC7	49	M		MN	MN	MN	MN	MN	MP	MN	MN	MP	MN
				MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
IP1	71	F		MN	MN	MP	MN	MN	MN	MN	MN	MN	MN
IP2	79	F		MN	MN	MN	MN	MN	MN	MN	MN	MN	MP
IP3	76	M		MN	MP	MN	MN	MN	MN	MN	MP	MN	MN
IP4	75	F		MN	MN	MN	MP	MN	MP	MP	MN	MP	MN
IP5	84	F		MN	MN	MN	MN	MN	MN	MP	MN	MN	MN
IP6	52	M		MN	MN	MP	MN	MN	MN	MP	MP	MN	MN
IP7	62	M		MN	MN	MN	MP	MN	MN	MN	MP	MP	MN
IP8	63	M		MN	MN	MP	MN	MN	MN	MN	MP	MN	MN
				MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
PC1	66	F	ⅡA	MP	MN	MN	MN	MN	MN	MN	MP	MN	MN
PC2	54	F	Ⅲ	MN	MP	MN	MN	MN	MN	MN	MN	MN	MN
PC3	66	M	ⅡB	MP	MP	MN	MN	MN	MN	MP	MN	MP	MP
PC4	67	M	ⅡB	MP	MN	MN	MN	MP	MN	MN	MN	MP	MN
PC5	71	M	ⅡB	MN	MP	MP	MN	MN	MN	MN	MN	MN	MN
PC6	49	F	ⅡA	MP	MP	MN	MN	MN	MN	MP	MN	MP	MP
PC7	73	F	ⅡB	MP	MN	MN	MN	MN	MP	MP	MN	MN	MN
PC8	75	M	Ⅲ	MP	MP	MN	MN	MN	MN	MN	MN	MP	MN
PC9	59	M	ⅡB	MN	MN	MN	MN	MP	MN	MP	MP	MP	MP
PC10	78	F	ⅡA	MP	MP	MP	MN	MN	MN	MP	MP	MN	MP
PC11	75	F	ⅡB	MN	MN	MN	MP	MN	MN	MP	MN	MN	MN
PC12	66	M	Ⅳ	MP	MP	MP	MN	MN	MN	MP	MN	MP	MP
PC13	70	F	Ⅳ	MP	MN	MN	MN	MN	MP	MP	MP	MP	MP
PC14	68	M	ⅠB	MN	MP	MN	MN	MN	MN	MN	MP	MN	MN
PC15	62	M	ⅡB	MP	MN	MP	MN	MN	MN	MN	MN	MN	MN

NC: Non-cancer; IP: Intraductal papillary and mucinous pancreatic tumour; pancreatic cancer (PC) 1, PC5, PC7, PC8 and PC13 are identical in Tables 1 and 2. MP: Methylation-positive; MN: Methylation-negative.

creatic cancer patients were compared with regard to the methylation patterns of 10 tumor-associated genes ($n = 5$). The methylation patterns were similar in both the pancreatic and biliary fluids from the same patients (Tables 1 and 2). The methylation patterns of the *UCHL1* and *RUNX3* genes were identical in the pancreatic and biliary fluids from the same patients.

Reactivation of UCHL1 expression by 5-AZA-dC/Trichostatin A treatment in pancreatobiliary cancer cell lines

To further examine the role of CpG methylation and histone deacetylation in silencing UCHL1, cancer cells were treated with 5-AZA-dC and/or TSA. 5-AZA-dC restored UCHL1 expression, and combined treatment with 5-AZA-dC and TSA restored UCHL1 expression synergistically at the mRNA level in pancreatobiliary cancer cell lines (Figure 4 and data not shown). TSA alone did not restore UCHL1 expression in cell lines.

DISCUSSION

In the present study, we found that the levels of methylation of LINE-1 were reduced in pancreatobiliary cancers compared to those in noncancerous pancreatobiliary disease. Genome-wide hypomethylation is known to be a common feature of human cancer, and genome-wide hypomethylation has recently been studied in various human malignancies using LINE-1 and other repetitive sequences as surrogates. Our results suggest that pancreatobiliary cancers exhibit a pattern of genome-wide hypomethylation that can be detected using pancreatic and biliary fluids.

Genome-wide hypomethylation is thought to be associated with tumor malignancy through a variety of mechanisms. For example, global hypomethylation is associated with genomic instability[32], which may confer a poor prognosis. Hypomethylation can also lead to the activation of proto-oncogenes, endogenous retroviruses or transposable elements; such transcriptional dysregulation could affect tumor aggressiveness. Although we found correlations between the level of LINE-1 methylation and the methylation of other repetitive sequences[17], it is possible that there are functional and/or biological differences in the regulation of repetitive DNA sequences. Further analysis is necessary to clarify the role of genome-wide hypomethylation in pancreatobiliary cancers.

The LINE-1 hypomethylation in pancreatic cancers determined using pancreatic fluids was less significant than that determined using tissue samples, although the

Table 2 CpG islands hypermethylation of tumor-associated genes in biliary fluids

	Age (yr)	Sex	Stage	*UCHL1*	*RUNX3*	*CDKN2A*	*IGF2*	*CACNA1G*	*AHRR*	*SFRP1*	*MGMT*	*APC*	*NEUROG1*
NC8	77	M		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC9	69	F		MN	MN	MN	MN	MN	MN	MP	MN	MN	MN
NC10	76	M		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC11	58	M		MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
NC12	41	M		MN	MN	MN	MN	MN	MP	MN	MP	MN	MN
NC13	66	M		MN	MN	MN	MN	MN	MP	MP	MP	MP	MP
NC14	69	F		MN	MN	MN	MN	MN	MP	MP	MP	MN	MP
NC15	71	F		MN	MN	MN	MN	MN	MN	MN	MP	MP	MN
NC16	59	M		MN	MN	MN	MN	MN	MP	MP	MN	MN	MN
NC17	54	F		MN	MN	MN	MN	MN	MN	MN	MN	MP	MP
NC18	67	M		MN	MN	MN	MN	MN	MP	MP	MN	MN	MN
NC19	85	M		MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
NC20	73	F		MN	MN	MN	MN	MN	MN	MP	MN	MN	MN
NC21	80	F		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC22	74	F		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC23	52	M		MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
NC24	64	M		MN	MN	MN	MN	MN	MN	MP	MP	MP	MN
NC25	64	F		MN	MN	MN	MN	MN	MP	MP	MN	MN	MN
				MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
GB1	75	F	ⅢB	MP	MP	MP	MP	MP	MP	MN	MN	MN	MN
GB2	76	F	ⅢB	MN	MP	MP	MP	MP	MP	MN	MN	MP	MN
GB3	62	M	ⅢA	MP	MP	MP	MP	MN	MN	MP	MP	MN	MN
GB4	67	M	ⅣA	MN	MP	MN	MN	MN	MN	MN	MN	MN	MN
GB5	59	F	ⅢB	MP	MN	MP	MN	MN	MP	MP	MN	MP	MP
GB6	63	F	Ⅱ	MP	MN	MP	MN	MN	MP	MP	MN	MP	MP
GB7	77	M	Ⅰ	MN	MP	MP	MN	MN	MN	MP	MP	MN	MN
GB8	78	M	ⅢA	MP	MN	MP	MN	MN	MN	MP	MP	MN	MN
BC1	73	M	Ⅰ	MN	MP	MN	MP	MN	MP	MP	MN	MN	MN
BC2	80	F	ⅡA	MP	MP	MN	MN	MN	MP	MP	MP	MN	MN
BC3	71	M	ⅡB	MP	MN	MN	MN	MP	MP	MP	MN	MN	MN
BC4	75	M	Ⅲ	MP	MN	MN	MN	MN	MN	MP	MP	MN	MN
BC5	77	M	ⅡB	MN	MP	MN	MN	MN	MN	MN	MP	MN	MN
BC6	65	M	ⅡA	MP	MN	MP	MN	MN	MN	MN	MN	MN	MN
BC7	72	M	ⅡA	MP	MN	MN	MN	MN	MN	MP	MN	MN	MN
BC8	73	F	Ⅳ	MP	MP	MN	MN	MN	MP	MN	MN	MN	MN
BC9	76	F	ⅡA	MP	MP	MN	MP	MP	MP	MP	MN	MN	MN
BC10	74	M	ⅡB	MP	MN	MN	MN	MN	MP	MN	MP	MN	MP
BC11	66	M	Ⅲ	MP	MN	MN	MN	MN	MN	MN	MN	MP	MN
BC12	58	F	ⅡB	MN	MP	MN	MN	MN	MN	MN	MN	MN	MN
PC1	66	F	ⅡA	MP	MN	MN	MN	MN	MN	MP	MP	MN	MN
PC5	71	M	ⅡB	MN	MP	MP	MN	MN	MN	MN	MP	MN	MN
PC7	73	F	ⅡB	MP	MN	MN	MN	MN	MP	MN	MN	MN	MN
PC8	75	M	Ⅲ	MP	MP	MN	MN	MN	MN	MN	MP	MN	MN
PC13	70	F	Ⅳ	MP	MN	MN	MN	MN	MP	MP	MP	MP	MN
PC16	59	M	ⅡA	MP	MP	MN	MN	MP	MN	MN	MN	MN	MN
PC17	80	F	ⅡB	MP	MP	MP	MP	MN	MP	MP	MN	MN	MN
PC18	67	M	ⅠB	MN	MP	MN	MN	MN	MP	MN	MP	MN	MN
PC19	63	M	ⅡA	MP	MN	MN	MN	MN	MN	MN	MP	MP	MN
PC20	78	F	ⅡB	MN	MN	MP	MN	MN	MP	MN	MN	MN	MN

NC: Non-cancer; GB: Gallbladder cancer; BC: Biliary cancer; pancreatic cancer (PC) 1, PC5, PC7 and PC8 and PC13 are identical in Tables 1 and 2. MP: Methylation-positive; MN: Methylation-negative.

number of samples analyzed was limited. It is possible that some of the pancreatic fluid samples did not contain sufficient concentrations of cancer DNA[12]. Given the relatively poor diagnostic yield of cytology in this setting, a problem that is likely to be related to the highly scirrhous nature of pancreatic ductal adenocarcinomas, sample adequacy is likely to be one of the limiting factors in the molecular analysis of these samples[12]. Serum LINE-1 hypomethylation has been reported to be a potential prognostic marker for hepatocellular carcinoma[33]. It would be interesting to analyze serum LINE-1 methylation levels in patients with pancreatobiliary cancers.

CpG island hypermethylation of tumor-associated genes was detected at various frequencies in pancreatobiliary cancers using pancreatobiliary fluids. Although genome-wide hypomethylation and regional hypermethylation of 5' CpG islands are common features of neoplasias, the link between the two remains controversial[17]. In the current study, we did not find a significant correlation between 5' CpG island hypermethylation of tumor-associated genes and global hypomethylation.

Hypermethylation of the *UCHL1* gene was cancer-

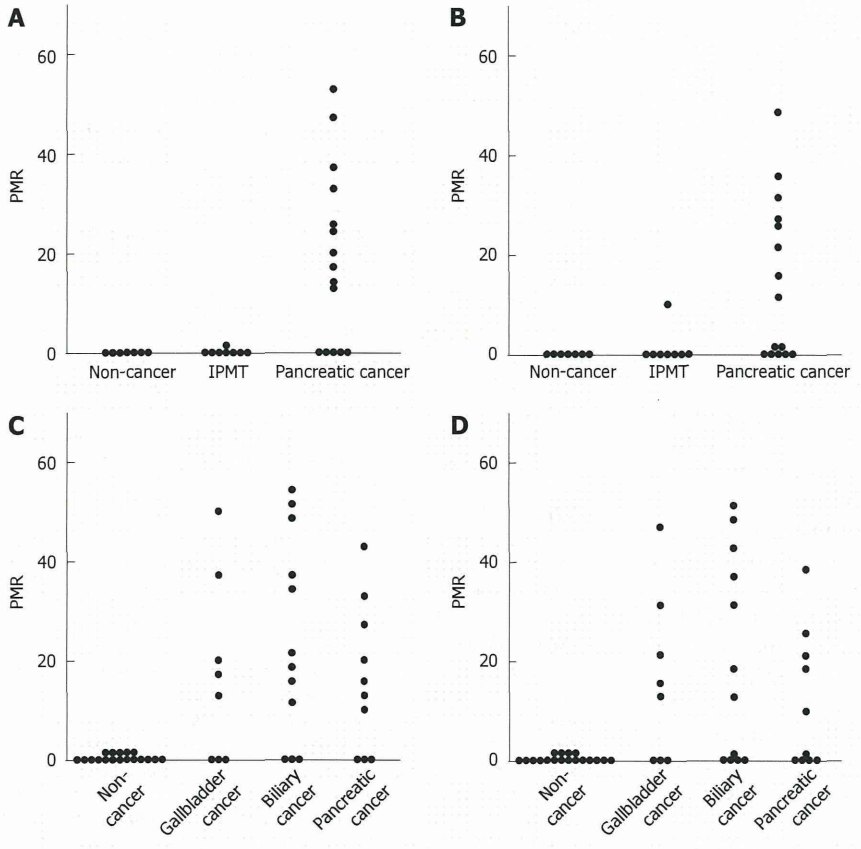

Figure 3 Analysis of methylation levels of ubiquitin carboxyl-terminal esterase L1 and runt-related transcription factor 3 using pancreatobiliary fluids. Comparison of the ubiquitin carboxyl-terminal esterase L1 (UCHL1) (A) and runt-related transcription factor 3 (RUNX3) (B) methylation levels in pancreatic fluids between pancreatic cancer and noncancerous pancreatic disease; Comparison of the UCHL1 (C) and RUNX3 (D) methylation levels in biliary fluids between pancreatobiliary cancer and noncancerous pancreatobiliary disease. PMR: Percentage of methylated reference.

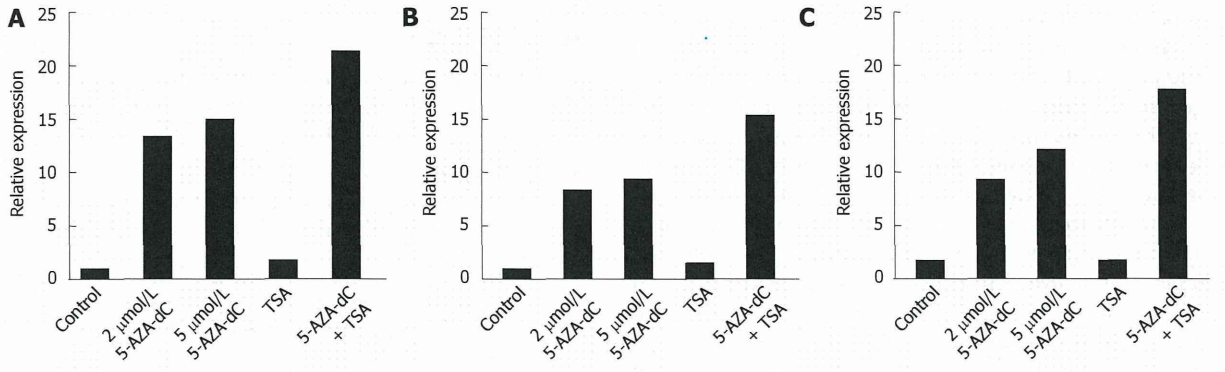

Figure 4 Reactivation of ubiquitin carboxyl-terminal esterase L1 by 5-AZA-2'-deoxycytidine and/or Trichostatin A treatment in pancreatic and biliary cancer cell lines. A: PK-1 cells; B: PK45P cells; C: TGBC1TKB cells. To examine the roles of CpG methylation and histone deacetylation in the silencing of ubiquitin carboxyl-terminal esterase L1, cancer cells were treated with 2 or 5 μmol/L 5-AZA-2'-deoxycytidine (5-AZA-dC) for 72 h or 100 nmol/L Trichostatin A (TSA) for 24 h. The cells were also treated with 2 μmol/L 5-AZA-dC for 72 h, followed by 100 nmol/L TSA for an additional 24 h.

specific and most frequently detected in pancreatobiliary cancers. Hypermethylation of the *UCHL1* gene in pancreatic and biliary fluids was the most useful single marker of pancreatic and pancreatobiliary cancers, respectively. Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic and biliary fluids was the most useful combined marker for pancreatic and pancreatobiliary cancers, respectively. Epigenetic inactivation of UCHL1 has been reported in a variety of human cancers[34]. Epigenetic inactivation of RUNX3 is known to play an important role in the pathogenesis of pancreatobiliary cancer[35,36].

LINE-1 and SAT2 methylation levels have been reported to be significantly lower in extrahepatic cholangiocarcinoma than in normal duct and biliary intraepithelial

neoplasias (BilINs). BilINs showed a decrease of SAT2 methylation levels, but no decrease of LINE-1 methylation levels was found compared to those in normal samples[24]. Most of the cancer-specific CpG island hypermethylation is thought to occur in the BilIN stage, before LINE-1 hypomethylation. Our results also suggest that CpG island hypermethylation analyzed in pancreatobiliary fluids is more useful than LINE-1 methylation for the detection of pancreatobiliary cancer.

Importantly, the methylation patterns of 10 tumor-associated genes were similar in both the pancreatic and biliary fluids from the same patients with pancreatic cancer. Moreover, the methylation patterns of the *UCHL1* and *RUNX3* genes were identical in both the pancreatic and biliary fluids from the same patients. These results further support the notion that hypermethylation of UCHL1 and RUNX3 in pancreatobiliary fluids is a useful marker for the detection of pancreatobiliary cancer.

To confirm the role of epigenetic alterations in transcriptional repression of the *UCHL1* gene, we treated pancreatobiliary cancer cell lines, in which UCHL1 was methylated, with 5-AZA-dC alone or in combination with TSA. Treatment with 5-AZA-dC restored the UCHL1 expression in cancer cell lines. Moreover, combined treatment with 5-AZA-dC and TSA restored UCHL1 expression synergistically, indicating that CpG methylation and histone deacetylation play important roles in silencing the *UCHL1* gene.

Not only the clinical utility but also the pathobiological effects of nucleic acids in circulation (nucleosomes, DNA, RNA, microRNA *etc.*) are receiving increasing attention[37,38]. Further analysis is necessary to clarify the possible detrimental effects of nucleic acids in the tumor microenvironment, including the contribution of methylated DNA in pancreatobiliary fluids to disease progression.

In conclusion, our results suggest that hypermethylation of the *UCHL1* gene plays a key role in the pathogenesis of pancreatobiliary cancers and that detection of hypermethylation of UCHL1 and RUNX3 in pancreatobiliary fluids is useful for the diagnosis of these malignancies. Our MethyLight panel (UCHL1 and RUNX3) is simple and accurate for differentiating between neoplastic and non-neoplastic samples and compares favorably with other quantitative MSP panels and with the identification of mutant KRAS or telomerase, which have been used previously to differentiate between malignant and benign pancreatic samples[39,40]. Moreover, newer assays that can detect low concentrations of mutations in pancreatic juice[41], as well as novel assays and technologies, are likely to improve the detection of low concentrations of mutant DNA for cancer diagnosis in the future. Although we focused on epigenetic alterations in the current study, a combination of highly specific epigenetic and genetic markers might provide the best diagnostic utility.

COMMENTS

Background

Despite recent advances in diagnosis and treatment, the prognosis of patients with pancreatobiliary cancer is still poor. Elucidation of the biological characteristics of these carcinomas has become necessary to improve the prognosis of patients and to devise better treatment strategies.

Research frontiers

Roles of epigenetic alterations in pancreatobiliary cancer are receiving increasing attention. Two contradicting epigenetic alterations often coexist in cancer: global or genome-wide hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters. Long interspersed nuclear element-1 (LINE-1) methylation status and its relationship with the hypermethylation of CpG islands in pancreatic and biliary fluids taken from patients with pancreatobiliary cancer is not known.

Innovations and breakthroughs

This is the first study to report that pancreatobiliary cancers exhibit a pattern of genome-wide hypomethylation that can be detected using pancreatic and biliary fluids. CpG island hypermethylation of tumor-associated genes was detected at various frequencies. Hypermethylation of the ubiquitin carboxyl-terminal esterase L1 (*UCHL1*) gene may play a key role in the pathogenesis of pancreatobiliary cancers.

Applications

Hypermethylation of UCHL1 and runt-related transcription factor 3 in pancreatobiliary fluids might be useful for the diagnosis of pancreatobiliary cancers. A combination of highly specific epigenetic and genetic markers might provide the best diagnostic utility.

Terminology

LINEs are 6-8 kb long, GC-poor sequences encoding an RNA-binding protein and a reverse transcriptase/endonuclease; these sequences constitute approximately 20% of the human genome. LINE-1 elements are most abundant, and over half a million copies of these elements are present in the human genome; UCHL1, which is also known as PARK5/PGP9.5, is a member of the ubiquitin carboxy terminal hydrolase family targeting the ubiquitin-dependent protein degradation pathway. With both ubiquitin hydrolase and dimerization-dependent ubiquitin ligase activities, UCHL1 plays important roles in multiple cellular processes. UCHL1 is a tumor-suppressor gene that is inactivated by promoter methylation or gene deletion in several types of human cancers.

Peer review

The presence of such high amounts of methylated DNA in pancreatobiliary fluid is intriguing. The study has translational significance.

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P- Reviewer Mishra PK **S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN

Oncogene (2012) 31, 1988–1994

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SHORT COMMUNICATION

Silencing of Kruppel-like factor 2 by the histone methyltransferase EZH2 in human cancer

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The Kruppel-like factor (KLF) proteins are multitasked transcriptional regulators with an expanding tumor suppressor function. KLF2 is one of the prominent members of the family because of its diminished expression in malignancies and its growth-inhibitory, pro-apoptotic and anti-angiogenic roles. In this study, we show that epigenetic silencing of KLF2 occurs in cancer cells through direct transcriptional repression mediated by the Polycomb group protein Enhancer of Zeste Homolog 2 (EZH2). Binding of EZH2 to the 5′-end of KLF2 is also associated with a gain of trimethylated lysine 27 histone H3 and a depletion of phosphorylated serine 2 of RNA polymerase. Upon depletion of EZH2 by RNA interference, short hairpin RNA or use of the small molecule 3-Deazaneplanocin A, the expression of KLF2 was restored. The transfection of KLF2 in cells with EZH2-associated silencing showed a significant anti-tumoral effect, both in culture and in xenografted nude mice. In this last setting, KLF2 transfection was also associated with decreased dissemination and lower mortality rate. In EZH2-depleted cells, which characteristically have lower tumorigenicity, the induction of KLF2 depletion 'rescued' partially the oncogenic phenotype, suggesting that KLF2 repression has an important role in EZH2 oncogenesis. Most importantly, the translation of the described results to human primary samples demonstrated that patients with prostate or breast tumors with low levels of KLF2 and high expression of EZH2 had a shorter overall survival.
Oncogene (2012) **31,** 1988–1994; doi:10.1038/onc.2011.387; published online 5 September 2011

Keywords: epigenetics; histone methyltransferase; EZH2; KLF2

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Received 5 March 2011; revised 11 July 2011; accepted 28 July 2011; published online 5 September 2011

The Kruppel-like factor (KLF) family of proteins, also known as SP1-like, is made up of a set of transcription factors that are present in a wide range of organisms, in which they fulfill a range of cell differentiation and proliferation functions (Black *et al.*, 2001; Kaczynski *et al.*, 2003; Zhao and Meng, 2005; Bureau *et al.*, 2009). KLFs have Cys2/His2 zinc-finger domains that preferentially bind to GC-rich target sequences, where they can function as activators or repressors in a cell type- and promoter-dependent manner (Black *et al.*, 2001; Kaczynski *et al.*, 2003; Zhao and Meng, 2005; Bureau *et al.*, 2009). KLFs are also emerging as potential tumor suppressor genes owing to their roles in the inhibition of proliferation, migration and angiogenesis, and in the induction of apoptosis, senescence and adhesion (Black *et al.*, 2001; Kaczynski *et al.*, 2003; Zhao and Meng, 2005; Bureau *et al.*, 2009). One member of the family, KLF2, is particularly interesting. Although it is not known to undergo genetic disruption in human tumors, KLF2 expression is diminished in many malignancies, such as prostate (Duhagon *et al.*, 2010) and ovarian (Wang *et al.*, 2005) cancer. From a functional standpoint, KLF2 possesses tumor-suppressor features such as induction of cell quiescence (Buckley *et al.*, 2001), enhancement of DNA-damage-associated apoptosis (Wang *et al.*, 2005), inhibition of leukemia cell proliferation (Wu and Lingrel, 2004), anti-angiogenesis properties (Bhattacharya *et al.*, 2005), and the suppression of cell growth mediated by KRAS (Fernandez-Zapico *et al.*, 2010) and epidermal growth factor receptor (Kannan-Thulasiraman *et al.*, 2010). Thus, we wondered about the molecular basis of the loss-of-function defects of KLF2 in human tumorigenesis and so set out to study the possible role of oncogenic repressive mechanisms.

One of the most important systems for maintaining the heritable repression of genes is that of the Polycomb group proteins (Ringrose and Paro, 2004; Martin and Zhang, 2005). These Polycomb group proteins are often de-regulated in human cancer (Pasini *et al.*, 2004; Valk-Lingbeek *et al.*, 2004). The Enhancer of Zeste

Homolog 2 (EZH2) is a component of the Polycomb repressive complex 2, which also includes SUZ12 and EED, and represses gene transcription by trimethylation of Lys27 of histone H3 (H3K27) (Simon and Lange, 2008). EZH2 has the hallmarks of an oncogene, particularly in prostate and breast cancer, where elevated levels are found in the more advanced forms of the disease (Varambally *et al.*, 2002; Bracken *et al.*, 2003; Kleer *et al.*, 2003). Recently, EZH2 gain-of-function mutations have also been found in lymphomas (Yap *et al.*, 2011). Thus, one way by which EZH2 could promote transformation is by repressing tumor suppressor genes, exploiting its methyltransferase activity for lysine 27 of histone H3 (H3K27) (Simon and Lange, 2008). In this scenario, several genes with a growth-inhibitory function have recently been found to be targeted by EZH2 in cancer cells (Chen *et al.*, 2005; Beke *et al.*, 2007; Yu *et al.*, 2007, 2010; Fujii *et al.*, 2008). Thus, we examined whether KLF2 could also be a key tumor suppressor gene targeted for repression by EZH2 in human tumorigenesis.

To assess the putative role of EZH2 in KLF2 repression, we first transiently depleted the expression of EZH2 by RNA interference (RNAi) in MDA-MB-231 and MCF-7 (breast), PC3 and LNCaP (prostate), and U2OS (osteosarcoma) cancer cell lines (Figures 1a and b). All cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modied Eagle's medium supplemented with 10% fetal bovine serum. The *in vitro* transient transfection of short interfering RNAs (Qiagen, Valencia, CA, USA) was performed using Oligofectamine (Invitrogen, Carlsbad, CA, USA). We observed that the loss of EZH2 was associated with

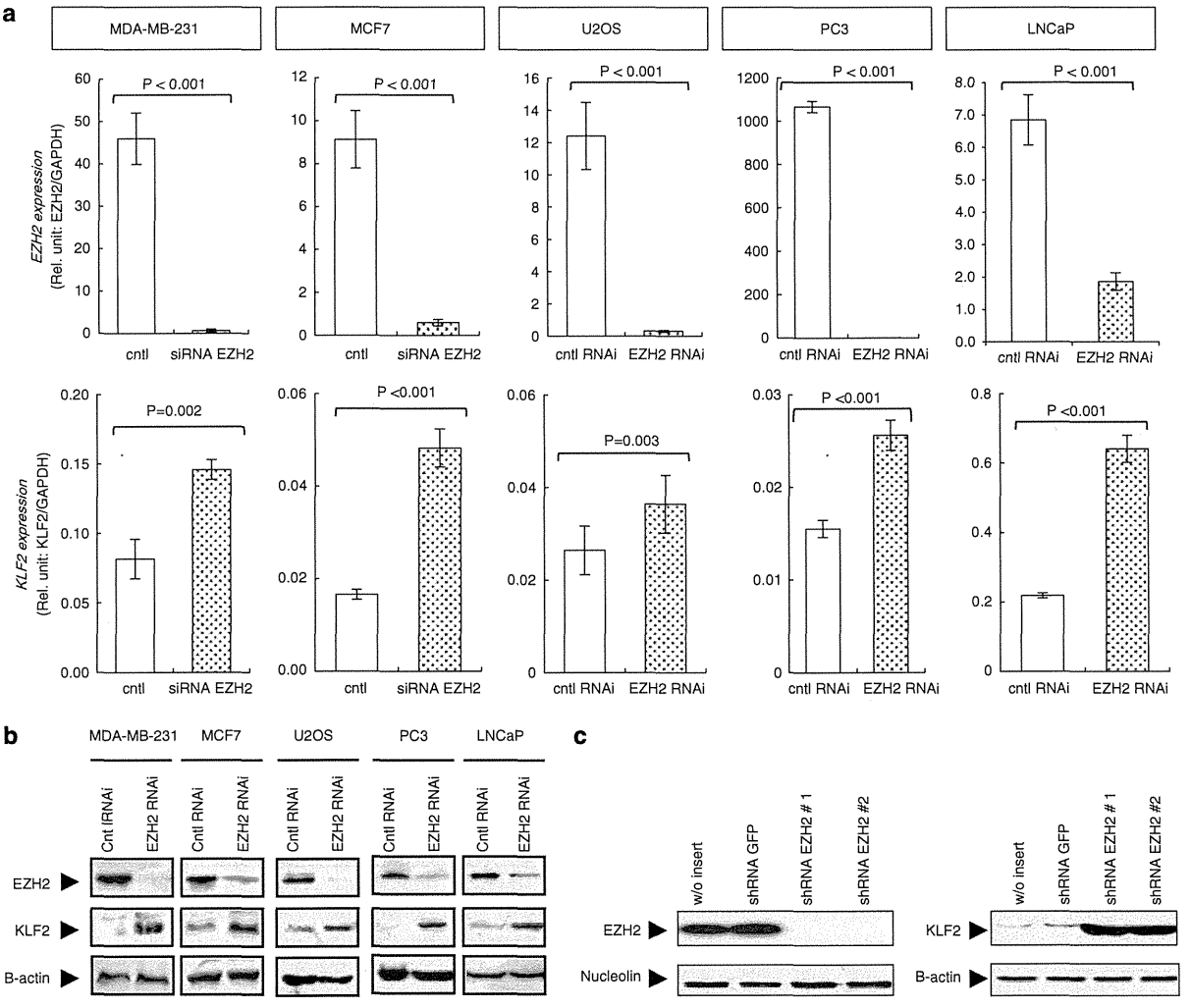

Figure 1 EZH2 depletion leads to increased KLF2 mRNA and protein levels. Expression of KLF2 and EZH2 determined by qRT–PCR (**a**) and (**b**) immunoblot in five cancer cell lines (U2OS, MDA-MB-231, MCF-7, PC3 and LNCaP cells) following transfection with oligo-type short interfering RNA (siRNA) against EZH2 or scrambled siRNA for 72 h as a transient model. (**c**) Immunoblot of EZH2 and KLF2 in stable EZH2 knockdown clones. U2OS cells are transfected with shRNA constructs targeting EZH2 or control vector and undergo selection with puromycin. *P*-values obtained from Student's *t*-test.