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*Biomarkers, Genomics, Proteomics, and Gene Regulation*

# Krüppel-Like Factor 10 Expression as a Prognostic Indicator for Pancreatic Adenocarcinoma

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**Deregulation of transforming growth factor (TGF)-β function is a common feature of pancreatic cancer, rendering these cancers unresponsive to TGF-β–stimulated growth inhibition. Recent findings have supported a primary role for Krüppel-like factor 10 (KLF10) as an important transcription factor involved in mediating TGF-β1 signaling. The aim of this study was to evaluate the correlation between KLF10 expression and the clinical and pathologic features of pancreatic cancer. Tissue specimens from patients with pancreatic adenocarcinoma were retrospectively collected for immunohistochemical analysis. To demonstrate that *Klf10* expression was primarily regulated by methylation status, the *Klf10* promoter was examined by methylation-specific PCR using a pancreatic cancer cell line (Panc-1). DNA methyltransferase (DNMT) inhibitor and small-interfering RNA depletion of *DNMT* genes were used to reverse KLF10 expression in the Panc-1 cells. In parallel, DNMT1 expression was evaluated in the pancreatic cancer tissue specimens. In 95 pancreatic cancer tissue specimens, KLF10 expression was inversely correlated with pancreatic cancer stage ($P = 0.01$). Multivariable analysis revealed that, in addition to the presence of distant metastasis at diagnosis ($P = 0.001$ and 0.001, respectively), KLF10 was another independent prognostic factor related to progression-free and overall survival ($P = 0.018$ and 0.037, respectively). The loss of KLF10 expression in advanced pancreatic cancer is correlated with altered methylation status, which seems to be regulated by DNMT1. Our results suggest that KLF10 is a potential clinical predictor for progression of pancreatic cancer.** ***(Am J Pathol 2012, 181: 423–430; http://dx.doi.org/10.1016/j.ajpath.2012.04.025)***

Pancreatic adenocarcinoma is one of the most lethal tumors across all cancer types, with only 10% to 15% of patients able to undergo curative intent surgery at the time of diagnosis.[1] Therefore, before the development of an effective therapy, it is urgent that biomarkers for early diagnosis are identified to differentiate patients with heterogeneous clinical outcomes.

Krüppel-like factor (KLF) family members share a three-$C_2H_2$ zinc finger DNA-binding domain with a Krüppel linker between the zinc fingers. These proteins are involved in cell proliferation and differentiation control in normal tissues.[2–5] Studies during the past several years have supported a significant role for this family of transcription factors in carcinogenesis.[6–8] KLF family members have been found to be deregulated in multiple cancers, which seems to occur by loss of heterozygosity, somatic mutation, or transcriptional silencing due to promoter hypermethylation.[9] Furthermore, the expression of these proteins has been shown to mediate growth inhibition when ectopically expressed in multiple cancer-derived cell lines. This occurs via the inhibition of a number of key oncogenic signaling pathways and leads to a reversal of the tumorigenic phenotype *in vivo*.[10–12]

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One Klf family member, the human homologue of *Klf10*, which is also called *TIEG1* [transforming growth factor TGF (TGF)-$\beta$ inducible early gene 1 at 8q22.2], was originally identified as the product of a TGF-$\beta$ inducible early-response gene in osteoblastic cells using differential display PCR. Further studies on the regulation by TGF-$\beta$1 of *Klf10* transcription have revealed that increased intracellular levels of KLF10 mimic the antiproliferative and apoptotic effects of TGF-$\beta$1 on epithelial cell growth, suggesting that Klf10 is an important factor mediating TGF-$\beta$1 signaling.[7]

The mechanisms regulating KLF10 and their biological significance have been confirmed in our previous studies.[11,13] Because pancreatic cancer is well known for being associated with a high incidence of TGF-$\beta$ signaling pathway deregulation, the aim of the present study was to investigate the correlation between KLF10 expression and the clinical features of pancreatic adenocarcinoma. In this study, the levels of KLF10 were measured by immunohistochemical analysis of pancreatic adenocarcinoma sections. An investigation was also performed to examine whether the decrease in Klf10 protein expression associated with advanced pancreatic cancer is under epigenetic control; it was found that a restoration of KLF10 expression in pancreatic cells occurred after treatment with the methyltransferase inhibitor 5-Aza-dC and after small-interfering RNA (siRNA) silencing of the DNA methyltransferase 1 (*DNMT1*) gene.

## Materials and Methods

### Cancer-Profiling Array

Hybridizations with a cancer profiling array (BD Biosciences, Franklin Lakes, NJ) using 25 ng of a gene-specific $^{32}$P-labeled cDNA probe, which was generated by labeling a *Klf10* cDNA fragment obtained by restriction digestion (1 to 210 bp) using an alkaline phosphatase end-labeling kit according to the manufacturer's recommended protocol (Abnova, Taipei, Taiwan). The tumor/normal intensity ratio was calculated using a Fuji-phosphorimager FLA5000 (Fuji, Japan) and normalized against the background.

### Patients and Tissues

We retrospectively reviewed 108 primary pancreatic adenocarcinomas from two institutions (National Taiwan University Hospital and National Cheng Kung University Hospital) that had been collected in 2003 to 2009. Of these cases, 95 had adequate tissue specimens and appropriate clinical data, and these cases constituted our study population. The clinical study was approved by institutional review board of both hospitals (National Cheng Kung University Hospital HR-99-033 and National Taiwan University Hospital 200804038R).

### Immunohistochemistry

Immunohistochemical staining was performed on 4-$\mu$m–thick, formalin-fixed, paraffin-embedded tissue sections. Mouse monoclonal antibody against human Klf10[11] and rabbit monoclonal antibody against human DNMT1 (NBP1-33609; Novus, St. Charles, MO) were used at dilutions of 1:500 and 1:225, respectively. After antigen retrieval by microwave pretreatment in 10 mmol/L citrate buffer of pH 6.0 for 10 to 20 minutes, the sections were cooled and incubated with the primary antibody (4°C overnight) and were detected using a MultiLink Detection Kit (QD200-QX; BioGenex, Fremont, CA). The peroxidase reaction was developed with diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin.

### Immunostaining Grading and Score

The tissue slides were examined independently by two observers (S.L.P. and P.Y.C.) who were masked to both the clinical and pathological data. Immunopositivity was assessed with respect to cellular localization, intensity, and distribution. Expression of Klf10 was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells graded on scale of 0 to 3: 0, none; 1, 1% to 30%; 2, 31% to 60%; and 3, >60%) and the intensity of staining (graded on a scale of 0 to 3: 0, none; 1, weak staining; 2, moderate staining; and 3, strong staining). The lesions were then jointly examined by both pathologists to resolve any discrepancies (<10%) in scoring. The combination of extent (E) and intensity (I) of staining was obtained by calculating E $\times$ I to give EI, which varies from 0 to 9. The mean EI score was calculated for each pancreatic cancer specimen. For statistical analysis, EI scores of 0 to 3 were considered low expression and EI scores >3 were considered high expression.

### Statistical Analysis

Survival curves were estimated using the Kaplan-Meier method,[14] and differences between the groups were analyzed using the log-rank test. Data on survivors were censored at the last follow-up. We used nonparametric tests to compare independent groups of numerical data (Mann-Whitney test) and categorical data ($\chi^2$ test and Fisher's exact test). Correlations among numerical variables were assessed by Spearman's rho correlation analysis. Multivariable analyses, with backward variable selection, were conducted using Cox's proportional-hazards regression model.[15] All of the statistical analyses were performed using the SPSS statistical software version 10.0 (SPSS Inc., Chicago, IL).

### Cell Culture, siRNA Transfection, 5-Aza-dC Treatment, and DNA Methylation Assays

PANC-1 cells were purchased and cultured according to the supplier's instructions (60284, Food Industry Research and Development Institute, Taipei, Taiwan; derived from ATCC: CRL-1469). Predesigned siRNAs against various DNMTs were purchased from National RNAi Core Facility (TRCN0000021891; Academia Sinica, Taipei, Taiwan) or from Santa Cruz Biotechnology (DNMT3a: sc-37757-SH; DNMT3b: sc-37760-SH; Santa Cruz Biotechnology, Santa Cruz, CA). The target sequence (5′-GCCCAATGAGACTGACATCAA-3′) was in-

**Table 1.** Methylation-Specific PCR Primers for Klf10

| Primers | Sequence | Primers | Sequence | Target sites (TCS = +1) |
|---|---|---|---|---|
| MSP-1-LM | 5′-TGAGGAAGTAGAATTCGGTGATC-3′ | MSP-1-LU | 5′-ATGAGGAAGTAGAATTTGGTGATTG-3′ | −709 to −523 |
| MSP-1-RM | 5′-GAACGCTACAAATAAAAAAAACGAA-3′ | MSP-1-RU | 5′-CAAACACTACAAATAAAAAAAACAAA-3′ | |
| MSP-2-LM | 5′-GGAGTTTGAGGTCGGTTAATTAAC-3′ | MSP-2-LU | 5′-GAGTTTGAGGTTGGTTAATTAATGG-3′ | −185 to −29 |
| MSP-2-RM | 5′-GAAACAAAACAAAAAAAACACGC-3′ | MSP-2-RU | 5′-CAAAACAAAACAAAAAAAACACACT-3′ | |
| MSP-3-LM | 5′-TTTATTTCGCGTTTTAGTGTTTTTC-3′ | MSP-3-LU | 5′-TATTTTGTGTTTTAGTGTTTTTTGA-3′ | −621 to −495 |
| MSP-3-RM | 5′-TAAAAACGCCATCCTCTTACG-3′ | MSP-3-RU | 5′-CATTAAAAACACCATCCTCTTACAC-3′ | |
| MSP-4-LM | 5′-TAATGTAAATTTTTTGGTTTCGG-3′ | MSP-4-LU | 5′-TAATGTAAATTTTTTGGTTTTGG-3′ | −496 to −198 |
| MSP-4-RM | 5′-AAACCACCTAATTAAATAACGCC-3′ | MSP-4-RU | 5′-AAAACCACCTAATTAAATAACACC-3′ | |
| MSP-5-LM | 5′-TTCGTAAGTATTCGGGAGGC-3′ | MSP-5-LU | 5′-TTGTTTGTAAGTATTTGGGAGGTG-3′ | 657 to 910 |
| MSP-5-RM | 5′-CAAAAAATCGTCAAAAAACGAC-3′ | MSP-5-RU | 5′-TCCAAAAAATCATCAAAAAACAAC-3′ | |
| MSP-5-1-LM | 5′-GTTCGTAAGTATTCGGGAGGC-3′ | MSP-5-1-LU | 5′-TTGTTTGTAAGTATTTGGGAGGTG-3′ | 656 to 911 |
| MSP-5-1-RM | 5′-CCAAAAAATCGTCAAAAAACG-3′ | MSP-5-1-RU | 5′-TCCAAAAAATCATCAAAAAACAAC-3′ | |
| MSP-6-LM | 5′-AGTAGTTAGTTAGTTTGTCGTCGGC-3′ | MSP-6-LU | 5′-GTAGTTAGTTAGTTTGTTGTTGGTGG-3′ | 114 to 278 |
| MSP-6-RM | 5′-TAATATCCGAAACTCGAAATTCG-3′ | MSP-6-RU | 5′-ACATAACTAATATCCAAAACTCAAAATTCA-3′ | |
| MSP-7-LM | 5′-GATTGGTCGGTTTTAGGTTTC-3′ | MSP-7-LU | 5′-ATTGGTTGGTTTTAGGTTTTGT-3′ | −49 to 156 |
| MSP-7-RM | 5′-ATAATTAACTACTTAACCGCCGA-3′ | MSP-7-RU | 5′-AATTAAACATAATTAACTACTTAACCACCA-3′ | |
| MSP-8-LM | 5′-GTTCGTAAGTATTCGGGAGGC-3′ | MSP-8-LU | 5′-TTGTTTGTAAGTATTTGGGAGGTG-3′ | 655 to 858 |
| MSP-8-RM | 5′-CAAACGTCTAAAAAAACAACTCGT-3′ | MSP-8-RU | 5′-CTCCAAACATCTAAAAAAACAACTCA-3′ | |
| MSP-9-LM | 5′-GTATCGTTTTTTTAGGTAGGGGC-3′ | MSP-9-LU | 5′-GTATTGTTTTTTTAGGTAGGGGTG-3′ | −290 to −137 |
| MSP-9-RM | 5′-ATAAAACGTATACACAATCCCCG-3′ | MSP-9-RU | 5′-ATAAAACATATACACAATCCCCAAC-3′ | |
| MSP-10-LM | 5′-TTGTGATTACGTATAAACGGTTAAAC-3′ | MSP-10-LU | 5′-TGATTATGTATAAATGGTTAAATGG-3′ | −1236∼−1022 |
| MSP-10-RM | 5′-ACGCCTAAAAATAAATTTACCGAC-3′ | MSP-10-RU | 5′-CACACCTAAAAATAAATTTACCAAC-3′ | |
| MSP-11-LM | 5′-TTTAGGCGTAGTATACGGGC-3′ | MSP-11-LU | 5′-AGTTTTTAGGTGTAGTATATGGGTG-3′ | −1147 to −856 |
| MSP-11-RM | 5′-CTACGAAATCGAATTTAAACTCGAT-3′ | MSP-11-RU | 5′-ACTACAAAATCAAATTTAAACTCAAT-3′ | |
| MSP-12-LM | 5′-TTTTACGTTGTCGGGTAGTC-3′ | MSP-12-LU | 5′-TAGTTTTATGTTGTTGGGTAGTT-3′ | −127 to 99 |
| MSP-12-RM | 5′-TTAACCACAAACGAACGC-3′ | MSP-12-RU | 5′-CTTAACCACAAACAAACACAC-3′ | |
| MSP-13-LM | 5′-GGGTAGTCGTCGATTACGC-3′ | MSP-13-LU | 5′-GGGTAGTTGTTGATTATGT-3′ | −115 to 101 |
| MSP-13-RM | 5′-ACTTAACCACAAACGAACGC-3′ | MSP-13-RU | 5′-ACTTAACCACAAACAAACAC-3′ | |
| MSP-14-LM | 5′-ACGATTTTTTGGAAGAGGC-3′ | MSP-14-LU | 5′-TTGATGATTTTTTGGAAGAGGT-3′ | 900 to 1135 |
| MSP-14-RM | 5′-TCTCTCCCTATAAACGCGAC-3′ | MSP-14-RU | 5′-CTTAACCACAAACAAACACAC-3′ | |
| MSP-15-LM | 5′-TGGTATCGAGGAGAGATGTTC-3′ | MSP-15-LU | 5′-TTTGGTATTGAGGAGAGATGTTT-3′ | 596 to 831 |
| MSP-15-RM | 5′-CAAAACTATCGACGAACCCT-3′ | MSP-15-RU | 5′-CCAAAACTATCAACAAACCCTC-3′ | |
| MSP-16-LM | 5′-GTTTTTCGTAGTGTTTGGGC-3′ | MSP-16-LU | 5′-TTTTTTGTAGTGTTTGGGTG-3′ | −341 to −137 |
| MSP-16-RM | 5′-ATAAAACGTATACACAATCCCCG-3′ | MSP-16-RU | 5′-AATAAAACATATACACAATCCCCAAC-3′ | |
| MSP-17-LM | 5′-GGGTAGTCGTCGATTACGC-3′ | MSP-17-LU | 5′-GGGTAGTTGTTGATTATGT-3′ | −115 to 89 |
| MSP-17-RM | 5′-ACGAACGCACGAAAACAC-3′ | MSP-17-RU | 5′-ACAAACACACAAAAACAC-3′ | |
| MSP-17_1-LM | 5′-ACGTTGTCGGGTAGTCGTC-3′ | MSP-17_1-LU | 5′-TTTATGTTGTTGGGTAGTTGTT-3′ | −123 to 89 |
| MSP-17_1-RM | 5′-ACGAACGCACGAAAACAC-3′ | MSP-17_1-RU | 5′-AAACAAACACACAAAAACACT-3′ | |

TCS, translational code star.

troduced into the pLKO.1-puro vector. The siRNA (100 nmol/L) per $1 \times 10^6$ cells were transfected using JetPEI transfection reagent from Polyplus transfection (Polyplus, New York, NY), following the manufacturer's instructions. Approximately 95% of the cells were efficiently transfected using a Cy3-conjugated siRNA, as ascertained by fluorescent microscopy. The resulting lysates were separated with 10% SDS-PAGE, transferred to Immobilon, and reacted with anti-DNMT1 antibody (NB100-264; Novus) or anti-actin antibody (NB600-532; Novus). After washing, the blots were incubated with an appropriate horseradish peroxidase–conjugated secondary antibody (Abcam, Cambridge, MA) and then reacted with ECL reagents before exposure.

The pancreatic cancer cell line, Panc-1, was also treated with 5-Aza-dC (Sigma-Aldrich, St. Louis, MO) for 2 days. Control cells were cultured with diluent buffer alone. Cellular RNA was extracted using Trizol reagent (Invitrogen, Grand Island, NY) with DNase I treatment. The mRNA levels of the *Klf10* and glyceraldehyde-3-phosphate dehydrogenase genes were assessed using TaqMan gene expression assays from Applied Biosystems (Carlsbad, CA) as recommended by the manufacturer. The amount of *Klf10* mRNA was normalized against the levels of glyceraldehyde-3-phosphate dehydrogenase.

To distinguish the methylation status of a sequence, methylation-specific PCR (MSP) was performed using differential chemical modification of cytosine residues in DNA. Probable methylated regions and PCR primers (Table 1) were obtained by analyzing the *Klf10* promoter sequence using MethPrimer software (Li Lab, University of California-San Francisco, San Francisco, CA),[16] a program for designing bisulfate conversion-based methylation PCR primers.

For the methylation assay, genomic DNA was isolated from Panc-1 and modified by sodium bisulfate using an EZ DNA Methylation Kit (Zymo Research, Irvine, CA). The bisulfite-modified DNA was then subjected to PCR amplification, resulting in PCR products covering the area −1234 to + 910 within the promoter and/or exon 1/intron 1 of *Klf10*. These CpG islands of the *Klf10* gene were amplified from the bisulfate modified genomic DNA by specific primers (Table 1). For the PCR amplification, a total volume of 10 $\mu$L was used, which contained the modified genomic DNA and 0.5 $\mu$mol/L of each primer. Each PCR product was then cloned using a TA Cloning Kit (Promega, Madison, WI).

## Results

### Expression of Klf10 mRNA in Normal Tissue and in Tumors from Various Organs

To demonstrate *Klf10* expression status in tissue from various organs, we examined commercially available ar-

rays of cDNAs made from pools of mRNAs obtained from individual tumors and their matching normal tissue. The results are presented in Figure 1. A lower level of Klf10 mRNA was noted in most tumors and in some of the normal tissues. *Klf10* was generally down-regulated in tumors obtained from the pancreas, kidney, and colon when compared with their normal counterparts. The presence of significant suppression of *Klf10* mRNA expression in the pancreatic cancer samples led us to conduct further investigations using clinical pancreatic specimens.

## *Klf10 mRNA Data Analysis from a cDNA Array File of Pancreatic Cancer*

We interrogated a published tumor transcriptome data set from the University of California, San Francisco, that consisted of 27 patients with pancreatic ductal adenocarcinoma to establish the transcript levels of *Klf10* in these tumors.[17] It was found that tumors with a lower level of *Klf10* expression had a shorter overall postoperative survival compared with those with a higher expression level (Supplemental Figure S1 at *http://ajp.amjpathol.org*).[16] However, the survival difference did not reach statistical significance (log-rank test $P = 0.152$), probably because of the small sample size of this cohort.

## *Patient Characteristics*

In total, 95 patients were enrolled in this study. The male/female ratio was 52:43, and 70 patients were younger than 70 years. Sixty-six patients (69.5%) presented with tumor over the pancreatic head. Twenty-seven patients (28.4%) had an initial serum CA19-9 level >400 U/mL. There were 8, 58, 12, and 17 patients who presented with stage I, II, III, and IV pancreatic cancer, respectively.

## *Immunohistochemical Analysis of KLF10 Expression in Pancreatic Cancer*

The immunostaining pattern of KLF10 is shown in Figure 2. KLF10 expression occurred preferentially within the cytosol of a normal pancreas, including ductal, acinar, and islet cells and pancreatic cancer tissue (Figure 2). The intensity of KLF10 staining is similar between major ductal cells and acinar cells in the normal pancreas (Figure 2, A and B). In general, the extent and intensity of staining were found to be more prominent in the normal pancreas compared with within cancer tissue (Figure 2C). In the nontumor part of pancreatic cancer, dilated major ducts and hyperplastic islet cells usually stain stronger than tumor cells (Figure 2C). The tumor specimens expressed a wide range of KLF10 intensity ranging from 1+ to 3+ (Figure 2, D–F). A correlation of the immunohistochemical staining results with the patients' clinical parameters was performed, and the results are presented in Supplemental Table S1 (available at *http://ajp.amjpathol.org*). For the 17 patients with stage IV cancer and the 9 patients with unresectable disease, imaging criteria of T and N stages were used in statistical analysis. Because of the amount and quality of specimens especially in stage IV disease, missing data of perineural or lymphovascular invasion were noted in nearly half of the patients. A high EI score for KLF10 was noted in 34.8% versus 10.3% of the patients with stage I/II versus III/IV disease. When the clinical parameters were considered,

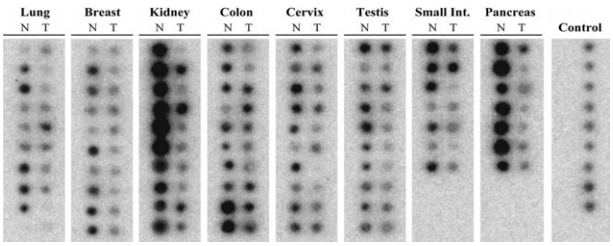


**Figure 1.** *Klf10* mRNA is differentially expressed in matched normal and tumor tissue samples. The cancer-profiling array contains pairs of cDNAs generated from matched tumor and normal tissue samples that were obtained from various different patients and spotted side by side on a nylon membrane. The array was hybridized with an $\alpha$-$P^{32}$-labeled probe for Klf10. Hybridization signals were detected by phosphorimaging, which was followed by scanning and manipulation with Adobe Photoshop. The analyzed tissues are indicated. N, normal; T, tumor. All of the samples on these arrays were normalized against a housekeeping gene, *ubiquitin cDNA* (control).

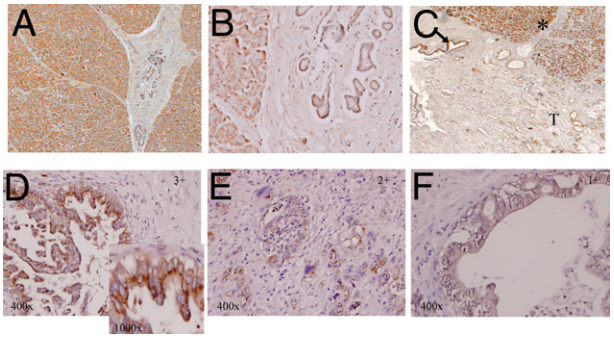


**Figure 2.** Immunohistochemical staining of human pancreatic normal and malignant tissues using anti-KLF10 monoclonal antibody. **A–B:** Normal pancreatic tissue. **C:** Junction between pancreatic ductal carcinoma and normal pancreas. T, tumor. The **asterisk** marks the normal pancreas; **arrow** indicates dilated normal duct. **D–F:** Representative Klf10 immunostaining of pancreatic carcinoma that shows weak (**F**), moderate (**E**), and strong (**D**) intensity. Original magnification: ×100 in (**A, C**), ×400 (**B**, **D–F**), and ×1000 (**D**, **inset**).

**Table 2.** Univariate Analysis of KLF10 Immunostaining Intensity

| Characteristic | *P* value | |
|---|---|---|
| | PFS | OS |
| Age of <70 vs ≥70 years | 0.92 | 0.15 |
| Male vs female | 0.32 | 0.45 |
| Location head vs other | 0.14 | 0.02 |
| CA19-9 <400 vs ≥400 U/mL | 0.01 | 0.15 |
| Stage I/II vs III/IV | 0.04 | 0.000 |
| Tumor stage I/II vs III/IV | 0.43 | 0.50 |
| Lymph node No vs N1 | 0.48 | 0.59 |
| Metastasis M0 vs M1 | 0.000 | 0.000 |
| Perineural invasion positive vs negative | 0.17 | 0.70 |
| Lymphovascular invasion positive vsnegative | 0.61 | 0.72 |
| Progression positive vs negative | | 0.23 |
| Surgery yes vs no | 0.002 | 0.000 |
| KLF10 EI low vs high | 0.007 | 0.02 |

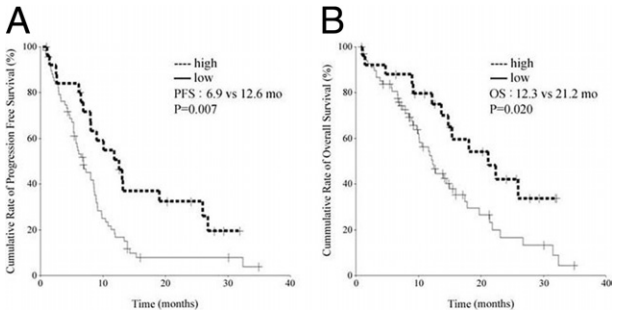


**Figure 3.** Survival curves of the pancreatic cancer patients. Kaplan-Meier survival curves of the pancreatic cancer patients ($n$ = 95) with high versus low KLF10 EI scores. **A:** PFS. **B:** OS. The $P$ value from the log-rank test is shown in each panel.

KLF10 expression was significantly correlated with tumor stage ($P$ = 0.01), metastasis ($P$ = 0.03), and surgery ($P$ = 0.03). Loss of KLF10 immunolabeling paralleled the advancement of the pancreatic cancer stage (see Supplemental Figure S2 at *http://ajp.amjpathol.org*; $P$ = 0.01), although the correlation was not strong (correlation coefficient = −0.299).

## *Prognostic Significance of KLF10 in Pancreatic Cancer*

Univariable analysis revealed that, besides serum CA19-9, surgery, stage, metastasis, and expression of Klf10 were significant predictors of progression-free survival (PFS) for pancreatic cancer patients (Table 2). A longer PFS was associated with higher KLF10 expression (Figure 3A, $P$ = 0.007). After including all potential prognostic factors in a multivariable analysis, only distant metastasis ($P$ = 0.001) and KLF10 expression ($P$ = 0.018) were predictive of PFS among pancreatic cancer patients (Table 3). When overall survival (OS) was considered, univariable analysis revealed that, other than stage, metastasis, location, and surgery, KLF10 expression was predictive of OS (Table 2, $P$ = 0.02). Patients with higher KLF10 expression had longer OS (Figure 3B, $P$ = 0.02). With the use of multivariable analysis, only young age ($P$ = 0.004), absence of metastasis ($P$ = 0.001), and higher KLF10 expression ($P$ = 0.037) were independently associated with a longer survival time (Table 3).

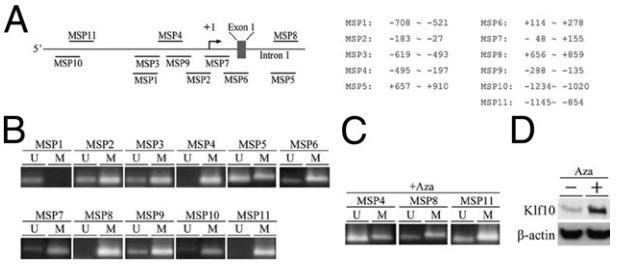


**Figure 4.** Methylation status of the *Klf10* promoter in a pancreatic cancer cell line. **A:** Maps of the *Klf10* gene promoter region and details of the CpG islands that were used for analysis of promoter methylation by several MSPs (MSP-1 to MSP-11). MSP was performed using methylation-specific and unmethylation-specific primer sets as detailed in Table 1. **B:** Hypermethylation of the *Klf10* promoter in the pancreatic cancer cell line (Panc-1). MSP was performed on bisulfite-treated DNA from the pancreatic cancer cell line. DNA bands in the various lanes that are labeled with U indicate PCR products amplified with primers recognizing an unmethylated *Klf10* promoter sequence. DNA bands in the various lanes that are labeled with M represent amplification products generated with methylation-specific primers. **C:** MSP was performed using DNA from cells after treated with 1 $\mu$mol/L 5-aza-2′-deoxycytidine (+Aza). **D:** KLF10 immunoblotting of Panc-1 cells with or without treatment with 5-aza-2′-deoxycytidine (+Aza).

## *Influence of DNA Methylation on the Expression of Klf10 Genes*

To demonstrate that the loss of *Klf10* expression in pancreatic cancer is primarily through regulation of methylation status, the promoter of the *Klf10* gene was examined using methylation-specific PCR (Figure 4A) in the pancreatic cancer cell line Panc-1. As shown in Figure 4, bisulfate-modified DNA was amplified using paired methylation and nonmethylation primers (Table 1). The results revealed that probable sites of methylation were located in the following regions: −495 to −197 (MSP-4), +656 to +859 (MSP-8), and −1145 to −854 (MSP-11) (Figure 4B).

To demonstrate a direct association between *Klf10* promoter methylation and loss of *Klf10* mRNA transcription expression, we treated Panc-1 with the demethylating agent 5-aza-dC. The intensity of the demethylated bands obtained from the cells treated with 5-aza-dC was significantly increased (Figure 4C). Furthermore, Klf10 protein expression in the Panc-1 cells was significantly increased by 5-aza-dC treatment (Figure 4D).

5-aza-dC is a global DNMT inhibitor. It has been reported that many cancer cells show a higher level of DNMT activity, which is believed to contribute to CpG island hypermethylation of tumor suppressor genes. In this study, DNMT1 protein expression was hardly notice-

**Table 3.** Multivariate Analysis of KLF10 Immunostaining Intensity

| | PFS | | OS | |
|---|---|---|---|---|
| Characteristic | $P$ value | Hazard ratio (95% CI) | $P$ value | Hazard ratio (95% CI) |
| Age | 0.149 | | 0.004 | 2.70 (1.37 – 5.34) |
| Location | 0.798 | | 0.800 | |
| Surgery | 0.079 | 0.48 (0.21 – 1.09) | 0.057 | 0.38 (0.14 – 1.03) |
| Stage | 0.073 | 0.42 (0.16 – 1.08) | 0.409 | |
| Metastasis | 0.001 | 4.01 (1.72 – 9.34) | 0.001 | 4.75 (1.97 – 11.46) |
| KLF10 EI score | 0.018 | 0.49 (0.27 – 0.88) | 0.037 | 0.46 (0.22 – 0.95) |

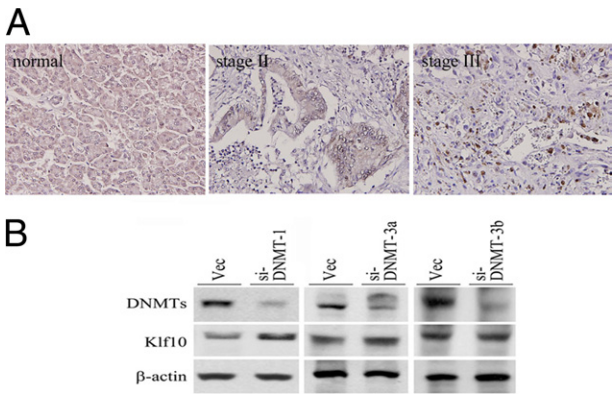


**Figure 5.** DNMT1 contributes to *Klf10* promoter methylation and protein expression. **A:** Immunohistochemical analysis of DNMT1 in human pancreatic normal and tumor tissues from various stages of pancreatic cancer (as indicated). Sections were counterstained with hematoxylin after immunostaining. Representative images were taken at ×400 magnification. **B:** Transient transfection of Panc-1 cells with various siRNA vectors targeting *DNMT1, DNMT3A,* and *DNMT3B.* In the upper panel, the siRNA vectors significantly knocked down expression of *DNMT1*, *DNMT3A*, and *DNMT3B* as expected. In the middle panel, expression of KLF10, as measured by Western blotting, was increased only after transient knockdown of DNMT1. Three independent experiments were performed. Approximately equal amount of proteins were loaded into each lane as indicated by their β-actin signals.

able in normal pancreatic tissue by immunohistochemical staining. However, overexpression of DNMT1 was observed in advanced pancreatic cancer specimens, mainly within the nuclei (Figure 5A). To address this question, we depleted different DNMT genes, including *DNMT1, DNMT3A, and DNMT3B,* using various siRNAs. The mRNA and protein levels of the various DNMTs were quantified after treatment by quantitative PCR and Western blot analyses. Using the same approach, we measured the effect of the various specific DNMT depletions on expression of *Klf10* genes in Panc-1 cells. Only depletion of DNMT1 was paralleled by an increasing level of Klf10 protein (Figure 5B).

## *Discussion*

Despite substantial advances in the diagnosis and treatment of pancreatic adenocarcinoma, it remains a disease with high morbidity and mortality. Therefore, novel molecular markers that help with early diagnosis and prognosis and with the development of therapeutic targets are urgently needed to help pancreatic cancer patients.

Among the many cytokines related to pancreatic cancer cell development, TGF-β1 is considered to be involved in many of the biological processes. However, the mechanisms underlying the effects of TGF-β1 on pancreatic cancer progression are currently poorly understood. KLF10 seems to play an important role in TGF-β1 signaling, which in turn regulates cell proliferation and apoptosis.[13,18] *In vitro* studies have implicated Klf10 as either a transcriptional activator or transcriptional suppressor, depending on the cell line examined.[19–21] Our laboratory and others have shown that KLF10 is a putative tumor suppressor.[11,22,23] Previous studies have demonstrated a close relationship between the expression levels of Klf10 and the clinical stage of breast cancer.[24] Overexpression of *Klf10* has been found to induce apoptosis in TGF-β1 sensitive pancreatic cancer cells by modulating stathmin, an important regulatory protein of microtubule dynamics.[25] In this study, we report that KLF10 expression is inversely correlated with pancreatic cancer stage ($P = 0.01$).

The significant correlation of KLF10 with the presence of distant metastasis at diagnosis ($P = 0.03$) reveals that loss of KLF10 during pancreatic carcinogenesis may be responsible not only for enhanced tumor cell proliferation and invasion but also for migration. After multivariable analysis, considering the influence of metastatic stage, KLF10 is still a significant prognostic factor for both PFS and OS among pancreatic cancer patients. These results imply that other independent mechanisms associated with KLF10 may contribute to the protein's prognostic significance in pancreatic cancer.

Increasing evidence has indicated that CA19-9 may serve as a prognostic marker for the preoperative evaluation of patients with pancreatic cancer.[26,27] In this study, KLF10 expression is not correlated with the serum level of CA19-9 ($P = 0.12$). However, statistical analysis reveals a highly significant role for KLF10 in predicting PFS and OS among pancreatic cancer patients compared with when serum CA19-9 is analyzed (Table 1). The clinical value of KLF10 in predicting pancreatic cancer progression, especially among patients with locally advanced disease, is worthy of further investigation.

*Klf10* is expressed in a range of organ tissues as described in Figure 1. In addition to epithelial cells, KLF10 has been reported to express inflammatory cells, to regulate T-regulatory cell suppressor function, and to be involved in $CD4^+CD25^-$ T-cell activation in association with TGF-β1 and Foxp3. $Klf10^{-/-}$ $CD4^+CD25^-$ T cells promote atherosclerosis by approximately twofold in *ApoE*$^{-/-}$*/scid/scid* mice, which is associated with increased leukocyte accumulation and higher levels of peripheral proinflammatory cytokines.[28] Furthermore, KLF10 is known to be strongly expressed in proangiogenic cells derived from bone marrow. *Klf10*$^{-/-}$ mice display reduced levels of circulating proangiogenic cells and possess multiple defects that are critical to neovascularization.[29] In our pancreatic cancer specimens, KLF10 was found to be expressed weakly in stromal fibroblasts, lymphocytes, and endothelial cells. The extent and intensity of KLF10 immunostaining in the stroma did not vary a lot across the different tumor stages of pancreatic cancer. The role of KLF10 in regulating tumor development and progression through changes in immune or hematopoietic function needs to be further investigated.

When the transcriptional regulation of signal molecules is explored, a number of human gene promoters have been found to contain dense stretches of the dinucleotide 5′-CpG-3′. These CpG islands serve as *cis*-regulation elements, may be methylated at the C5 position of cytosine, and tend to be associated with gene silencing. This DNA methylation at CpG residues has been known to be catalyzed by various DNMTs.[30–32] Among these methyltransferases, DNMT1 is present at high level and main-

tains DNA methylation by fully methylating hemimethylated CpGs. DNMT3a and DNMT3b are found at low levels in somatic cells and are catalytically slow enzymes. *DLC1*,[33] *Cdkn1c*,[34] and *DLEC1*,[35] which are specifically silenced by DNMT1, have shown promise as approaches to predicting cancer progression. Given the important role of DNMT1 in regulating gene methylation, depletion of this enzyme within cells should cause a significant decrease in genome-wide DNA methylation levels, which in turn ought to affect the transcriptome and the associated phenotypic characteristics of cells.[36–38] DNMT1 inhibitors, including 5-aza-dC and antisense-oligos, are currently under evaluation in various clinical trials.[39]

Our results strongly suggest that there is epigenetic regulation of *Klf10* by DNMT1 during pancreatic progression. However, the difficulties associated with obtaining sufficient and qualified tissue specimens from pancreatic cancer patients, especially from those who present with advanced disease, have prevented us from getting significant and reliable data in this area. Notwithstanding the results obtained in the present study, in addition to the effect of methyltransferase on *Klf10* expression, it is clear that other types of epigenetic modification of the *Klf10* promoter or even transcriptional/translational regulation of *Klf10* expression during pancreatic tumor progression cannot be excluded.

In summary, the current study indicates that methylation of the *Klf10* promoter region may alter expression of the *Klf10* gene and may therefore be one of the contributing factors to KLF10 inactivation during pancreatic cancer progression. Thus, our results support the idea that KLF10 plays an important role in regulating pancreatic cancer progression. Furthermore, KLF10 is a novel prognostic marker for pancreatic cancer and has potential as a therapeutic target when developing new therapies targeting pancreatic cancer.

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