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# Increased alternative splicing of the KLF6 tumor suppressor gene correlates with prognosis and tumor grade in patients with pancreatic cancer

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## Abstract

The aim of this study was to correlate the status of the KLF6 tumor suppressor gene including loss of heterozygozity (LOH), mutation and alternative splicing in human pancreatic cancer with tumor grade and survival.

Whereas neither KLF6 loss nor mutation was identified, expression of the KLF6 alternative splice forms was significantly increased in pancreatic tumor samples and cell lines. These cancers demonstrated marked cytoplasmic KLF6 expression, consistent with over-expression and accumulation of KLF6 splice form(s), which lack a nuclear localization signal. In addition, KLF6 splicing correlated significantly with tumor stage and survival.

In summary, pancreatic cancer displays a novel pattern of KLF6 dysregulation through selectively increased expression of KLF6 splice variants. Therefore, determination of KLF6 mRNA splicing levels may represent a novel biomarker predicting prognosis.

### Keywords

Pancreatic cancer; krüppel-like factor 6; tumor suppressor gene

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CONFLICT OF INTEREST

There are no conflict of interest to be declared by the authors of the manuscript.

## INTRODUCTION

Krüppel-like factor 6 (KLF6) is a member of the Krüppel-like factor family of transcription factors; this family contains at least twenty members, which are defined by their common 81 amino acid C-terminal DNA-binding domain (1–3). Krüppel-like factors exhibit a remarkable range of activities regulating cell growth and differentiation in virtually all tissues (4, 5). They may function either as transcriptional activators or repressors, depending on the cell type and promoter context (4, 5).

The KLF6 gene was originally cloned from human placenta (6) and activated rat hepatic stellate cells (7). Chromosomal deletion of the region containing the KLF6 locus (10p15) in prostate cancer (8), combined with its characterization as a growth suppressor, led to the identification of *KLF6* as a tumor suppressor gene frequently inactivated in prostate cancer (8). Growth suppressive mechanisms of KLF6 include transcriptional induction of p21 in a p53-independent manner (8), upregulation of TGFβ1 and its receptors (9), inactivation of *c-jun* (10), inhibition of other proto-oncogene signaling pathways (11), and sequestration of cyclin D1 (12). In addition to prostate cancer (8, 13), inactivation of KLF6 by loss and/or mutation has now been identified in several other cancers, including gastric (14), colorectal (15), hepatocellular (16), and ovarian carcinoma (17). In addition, downregulation of KLF6 mRNA has been identified in primary non small cell lung carcinoma (18), and reduced expression of KLF6 mRNA is associated with worse prognosis in prostate cancer (19).

We recently described an additional mechanism of KLF6 inactivation through the generation of alternative splice products of the *KLF6* gene that are over-expressed in prostate and ovarian cancers (17, 20). These splice forms inhibit the function of the wild-type, full length protein, even in the absence of *KLF6* loss or inactivating mutation (20). Mechanisms underlying generation of these splice forms in cancer have not been fully clarified, however a pathway in somatic cells has been identified in which presence of a G to A polymorphism in the first intron of the KLF6 gene leads to increased splicing that is associated with enhanced risk of prostate cancer (20). Moreover, splicing is correlated with Ras oncogene activation in hepatocellular carcinoma, which directly increases KLF6 splicing in cultured cells (21). Abrogation of splice form expression through use of specific siRNAs reduces cell growth in culture and prostate tumor xenografts, thereby confirming their growth-promoting, tumorigenic activity (17, 22). The net activity of KLF6 is thus represented by the relative expression of full length to splice form mRNA, which can be expressed simply as the ratio of KLF6wt/KLF6 splice form mRNA, as determined by both quantitative real time PCR and western blotting.

Carcinoma of the pancreas is among the most lethal of solid organ tumors. The disease typically presents late, when curative resection is not possible (23). Moreover, no specific biomarkers have been identified to enable early diagnosis (24). In addition, advanced pancreatic cancer is generally resistant to chemotherapy (25), and a response rate of only one-quarter or less can be expected with standard agents like gemcitabine (26). The molecular mechanisms underlying the development of chemotherapy resistance in pancreatic cancer are not clear.

In the present study we have examined *KLF6* gene expression in pancreatic cancer. Specifically, we have characterized the frequency of loss of heterozygosity (LOH), mutation and alternative splicing in 24 well-characterized primary pancreatic adenocarcinoma samples and correlated these findings with clinically relevant disease endpoints.

## MATERIALS AND METHODS

### Clinical data

Pancreatic cancer tissues were obtained from 24 patients (13 female, 11 male) undergoing a pylorus-preserving Whipple resection due to pancreatic ductal adenocarcinoma. In all patients an R0 resection was performed. Normal pancreatic tissues were obtained from 8 individuals (3 female, 5 male) through an organ donor program. Immediately following surgical removal, all tissue samples were either fixed in formaldehyde or frozen in liquid nitrogen. All cancer tissue samples were graded independently by a pathologist, and classified histologically as ductal adenocarcinoma of the pancreas. All clinical data in Berne and Heidelberg were registered in a prospective database between January 1995 and December 2003 (Table 1).

The median age of the patients undergoing a pancreaticoduodenectomy for pancreatic cancer was 68 years (range: 52–83 years). The median age of the normal pancreatic organ donors was 42 years (range: 25–50 years). According to the Classification of the UICC (International Union Against Cancer), there were four patients with stage I tumors, five with stage II, 14 with stage III, and one with stage IV tumors. Tumor grading (27) was well differentiated in 7 cases, moderately differentiated in 8 cases, and undifferentiated in 9 cases. The median survival in the group of patients with pancreatic carcinoma was 11 months (range: 8–27); (Table 2). The study was approved by the Ethics Committee of the University of Berne, Switzerland, and the University of Heidelberg, Germany.

### Pancreatic cancer cell lines

Seven human pancreatic carcinoma cell lines were used: The moderately differentiated human pancreatic adenocarcinoma cell lines T3M4, Capan-1, BxPC-3, and the less differentiated human pancreatic carcinoma cell lines AsPC-1, Colo-357, PANC-1, and Mia PaCa-2 were obtained from the American Tissue Type Culture Collection (Rockville, MD). Cell lines were cultured in Dulbecco Modified Eagle Medium (DMEM; Life Technology, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technology), penicillin G (100 units/mL), and streptomycin (100 μg/mL). Cells were grown as a monolayer culture at 37 °C in humidified air with 5% (Capan-1, Colo-357, AsPC-1, T3M4, BxPC-3) or 10% $CO_2$ (Mia PaCa-2 and PANC-1). Unless otherwise indicated, all chemicals were purchased from Sigma Chemicals (St. Louis, MO).

### Immunohistochemistry

Paraffin-embedded tissue sections (2–4 μm in thickness) were subjected to immunostaining using the Dako Envision + System (Dako Diagnostics AG, Zürich, Switzerland). Tissue sections for each tissue sample were deparaffinized with xylene and rehydrated through graded alcohol into distilled water. Endogenous peroxidase activity was quenched by

incubating the slides in 0.03 % hydrogen peroxide and sodium azide, followed by washing in Tris-buffered saline. The sections were then incubated overnight at 4°C with rabbit polyclonal antibody KLF6 (sc7158, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 0.05 mol/L Tris-HCL buffer containing 1% bovine serum albumin. KLF6 antibodies recognizing the amino terminus region (amino acids 28–201) conserved in all KLF6 splice forms were raised in rabbits. Bound antibody was detected with a streptavidin-biotin-horseradish peroxidase (HRP) system (DAKO Diagnostics AG) in which slides were successively incubated with biotinylated antirabbit IgG, streptavidin-HRP, and 3–3′diaminobenzidine (DAB). To ensure antibody specificity, control slides were incubated either in the absence of primary antibody or with a nonspecific IgG antibody; immunostaining was not detected in either case. All slides were analyzed by two independent observers blinded to patient status. Any differences in the findings were resolved by joint review and consultation with a third observer.

## Quantitative PCR for KLF6wt and KLF6sp in human pancreatic cancer tissue and cultered pancreas tumor cells

For quantifying target gene expression, RNA isolation from cultured cells and patient samples was performed using RNeasy Mini and Midi kits (Qiagen). All RNA was treated with DNase (Qiagen). RNA (1 μg) was reverse transcribed for each reaction using first-strand cDNA synthesis with random primers (Promega, Madison, WI). Real time PCR reactions were optimized to amplify either *KLF6* wild type alone (*KLF6wt),* or total *KLF6* mRNA (a primer designed and validated as previously described (20, 22) to detect both wtKLF6 and all KLF6 splice forms). Thus KLF6 splice form mRNA (*KLF6sp*) expression was calculated by determining the difference in absolute amount of these two PCR products. Thus, expression of *KLF6wt* (i.e., full length) mRNA and *KLF6total* mRNA (= *KLF6wt* + *KLF6sp*) were determined by quantitative real-time PCR using the following PCR primers on an ABI PRISM 7900HT Sequence Detection System (APPLIED Biosystems, Foster City, CA): *KLF6wt* forward 5’-CGG ACG CAC ACA GGA GAA AA-3’ and *KLF6wt* reverse 5’-CGG TGT GCT TTC GGA AGT G-3’; *KLF6total* forward 5’-CTG CCG TCT CTG GAG GAG T-3’ and *KLF6total* reverse 5’- TCC ACA GAT CTT CCT GGC TGT C-3’. All experiments were performed in triplicate and normalized to *GAPDH* mRNA expression. To calculate the fold change in *KLF6sp,* the fold change in total KLF6 was divided by the fold change in *KLF6wt* alone. The clinical data of the patients were correlated with the ratio of *KLF6wt/KLF6sp* mRNA expression. All quantitative real time PCR data shown in Results represent three independent real time PCR reactions that have each been performed in triplicate.

## Loss of KLF6 heterozygosity (LOH) analysis in human pancreatic cancer

Fluorescent LOH analysis was performed using genomic microdissected DNA from matched normal/pancreatic cancer as previously described (8). Fluorescently labeled microsatellite markers flanking KLF6 and ordered according to the Marshfield map were generated. PCR was performed according to manufacturer’s suggestions (Perkin Elmer, Boston, MA); markers D10S591 and D10S594 that flank the KLF6 gene, as well as three KLF6 specific markers, KLF6M1, KLF6M2, and KLF6M4. Primer sequences for KLF6 specific markers were as follows: KLF6M1 F: 5’ GAG GGA GTG AGG CTT TCT GTT 3’;

KLF6M1 R: 5’ TTT CCA GCC CAC TGT CTT CTT GAC 3’; KLF6M2 F: 5’ ATG GCC CTG ACT TCT 3’; KLF6M2 R: 5’TAC TTG CGG AGC GTG AGC C 3’; KLF6M4 F: GCA TTA AGA ATA GTG AAG GC 3’; KLF6M4 R: 5’ GAT GTG TTT GGC TCA GGG A 3’. The exponential range of the PCR was determined for each sample, and was between 30–38 cycles. The data were analyzed using the ABI Genescan and Genotyper software packages (Perkin Elmer) and allelic loss was scored by two independent observers as described before (16). In our system, a relative allele ratio of less than 0.7 was defined as loss of heterozygosity. The XLOH was confirmed at least twice for each marker. LOH analysis of the TP53 gene locus was performed as described above using three microsatellite markers flanking its locus: D17S796, D17S578 and D17S786.

### Western Blot

Pancreatic cancer cell lines (n=7), human pancreatic cancer tissues (n=7) and human normal pancreas tissue (n=5) were homogenized in ice-cold suspension buffer (10 mM Tris-HCL, pH 7.6, 100 mM NaCl) containing a complete protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The homogenized material was collected and centrifuged at 4° C for 30 minutes at 14000 × g to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay method (Pierce, Rockford, IL, USA). A total of 40 μg protein/lane was separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, blots were incubated with a polyclonal antibody against KLF6. After washing, blots were incubated with anti-rabbit IgG (Amersham International, Amersham, Bucks, UK) conjugated with horseradish peroxidase. Visualization was performed by the enhanced chemoluminescent method (Amersham International, Freiburg, Germany).

### Statistical Analysis

The age and survival of all patients are reported as median and range. Furthermore, we analyzed the median KLF6wt/sp ratio according to the four tumor stages (UICC) and tumor grades (I, II, III) (27). Differences between the groups were analyzed using Kruskal-Wallis and Mann-Whitney-U tests for non-parametric data with $p< 0.05$ considered statistically significant. For the relationship between survival analysis and KLF6wt/sp, log-rank test was used. Additionally, in a Cox-regression analysis we tested KLF6wt/sp ratio together with stage, and grade as continuous covariates for an independent prognostic factor versus survival. Correlation of KLF6wt/sp with tumor grading, tumor stage and survival was analyzed by the Spearmans test. Bivariate analysis was done by Mann-Whitney test.

## RESULTS

### Enhanced cytoplasmic KLF6 expression in pancreatic carcinoma

**Normal pancreas tissue—**We first assessed the pattern of KLF6 expression in normal pancreatic tissue by immunostaining of paraffin sections using a KLF6 polyclonal antibody (n=15). KLF6-immunoreactivity was present in scattered islet cells, and weak staining was also apparent in ductal cells. No KLF6 immunostaining was apparent in acinar cells or pancreatic micro-vessels (Figure 1A).

**Ductal adenocarcinoma of the pancreas—**In contrast to normal pancreas, ductal carcinoma cells displayed marked KLF6 immunoreactivity within the cytoplasm of tumor cells. Acinar cells and normal ducts within the cancer tissue were negative, and only some micro-vessels surrounding the tumor exhibited KLF6 immunoreactivity. Islet cells in the normal pancreas tissue within the cancer samples had similar staining as islets within normal controls. These findings were consistent among 15 samples analyzed, and a representative photomicrograph is shown (Figures 1B and 1C).

## KLF6 protein isoforms in normal pancreas and pancreatic carcinoma

Given the marked over-expression of cytoplasmic KLF6 in pancreatic tumor samples we sought to specifically define which KLF6 isoforms were present in both normal and cancerous pancreatic tissue. Full length wild-type KLF6 (wtKLF6) typically migrates on Western blot as a single or double band at ~46 kD, whereas alternative splice products have lower molecular weights, with the predominant splice form, SV1, detectable as a 26 kD protein (20). Accordingly, we examined both normal pancreas and carcinoma for the presence and relative expression of the various KLF6 isoforms present in these tissues. In normal pancreas only KLF6 wild type (46 kD) was detected. In contrast, in human pancreatic cancer tissue, bands of 40, 30, and 26 kD were identified in addition to KLF6. These lower MW bands are consistent with the previously described alternatively spliced products of KLF6 (20) (Figure 2).

## Loss of heterozygosity (LOH) analysis of KLF6

Our previous studies have demonstrated a widely variable frequency of LOH of the *KLF6* locus in human cancers, depending on the tumor type. For example, in prostate cancer, ~70% LOH was detected (8), whereas in hepatocellular carcinoma there was 39% LOH (16). Interestingly none of the pancreatic carcinomas analyzed displayed LOH of the KLF6 locus. To validate the methodology, findings for KLF6 were compared to LOH of the p53 locus, which was present in two of the seven samples (Figure 3). This frequency of LOH at the p53 locus is considerably lower than that reported recently (28), although p53 LOH is typically more common in invasive, non-resectable pancreatic cancers (29). Moreover, LOH detection is greatly increased by microdissection, which was not performed in our study (30). However, genetic divergence of pancreatic cancer with respect to tumor suppressor gene alterations is common (31).

## Increased KLF6 alternative mRNA splicing in pancreatic cancers and cell lines

We used quantitative real time PCR method to compare KLF6 splicing in normal and malignant pancreatic tissues. The expression of KLF6wt and total mRNA splice forms were analyzed in 24 pancreatic cancers and in 8 normal pancreatic tissues. The real time PCR data, together with clinical characteristics of the patients are presented in Table 1. As shown in Table 2, in normal pancreas the median KLF6wt/sp ratio was 3.45 (range: 2.71–4.95) compared to 2.08 in pancreatic tumors (range: 1.75–2.37) (Table 2) ($p=0.03$). The reduced ratio of KLF6wt/sp was almost entirely due to higher expression of KLF6sp in the cancer samples.

We also analyzed *KLF6* alternative splicing in pancreatic cancer cell lines (AsPC-1, T3M4, BxPC-3, MIA PaCa-2, PANC-1, Capan-1). In all cell lines examined, the ratio of KLF6wt to KLF6sp was consistently reduced (Figure 4): BxPC-3: 0.79, Capan-1: 0.85, PANC-1: 1, and AsPC-1: 1.02). KLF6wt/sp in pancreatic cancer cell lines was significantly lower than in primary human pancreatic cancer or normal pancreatic tissue ($p=0.003$ and $p=0.0003$, respectively; Figure 4). These data indicate that KLF6 alternative splicing is increased in pancreatic cancer cell lines even more than in primary pancreatic cancers.

### Correlation between KLF6wt/sp ratio and clinical data

We examined the relationships between the ratio of wild type to splice form mRNA (KLF6wt/sp) and both tumor grade and clinical data in patients with pancreatic cancer. In well-differentiated cancer samples (G1), the median ratio of KLF6wt/sp was 2.33 (range: 2.13–3.57), compared to 2.18 (range: 1.65–2.44) in moderately differentiated tumors, (G2), and 1.75 (range: 1.49–1.83) in poorly differentiated cancer samples (G3). KLF6wt/sp was significantly related to the tumor grade (G1-G3), with more poorly differentiated tumors having a lower KLF6wt/sp ratio than well-differentiated tumors ($p=0.001$; Table 2). Additionally, bivariate analysis demonstrated a significant difference between KLF6wt/sp >/< 2 and grading ($p<0.001$).

We also examined the relationship between the relative ratio of KLF6wt/sp as a function of disease stage according to UICC criteria. In UICC stage I, the median KLF6 wt/sp ratio was 2.95 (range: 2.18–3.57); (median survival: 28 months), in UICC II, 2.08 (range: 1.8–2.3); (median survival: median 14 months), and in UICC III, 1.84 (range: 1.48/2.3); (median survival: 10 months). There was only one patient with UICC IV who survived 11 months after surgery; this patient had a KLF6wt/sp ratio of 1.55. There was a trend towards correlation between KLF6wt/sp and tumor stage (UICC), but did not reach statistical significance ($p=0.076$) (Table 2).

However, the KLF6wt/sp ratio at the time of resection was highly correlated with patient survival ($p<0.001$). (Table 2) The bivariate anaylsis and the log-rank test revealed significantly longer survival in those patients with KLF6wt/sp ratio > 2 (median: 21 months; range: 14–19) than the survival of patients with a KLF6wt/sp ratio < 2 (median: 9 months; range: 6–10); ($p=0.005$) (Figure 5). Furthermore, the Cox-regression revealed KLF6 ratio as an independent marker for survival (p=0.006).

## DISCUSSION

In the present study we have characterized the KLF6 allele status and the expression of KLF6 mRNA in human pancreatic tumors and cancer cell lines. With the recent discovery that KLF6 mRNA is alternatively spliced in human prostate cancer (20), we focused on pancreatic cancer because of its highly lethal nature, our limited understanding of underlying mechanisms, and because of the availability of a very well characterized set of tumors associated with detailed clinical data, including survival. The findings build upon a substantial body of data implicating inactivation of KLF6 in the pathogenesis of a number of human cancers (8, 13, 15, 16), but provide new information regarding the prognostic value of KLF6 alternative splicing.

These data reinforce the potential role of enhanced alternative splicing of KLF6 as a growth-promoting mechanism in human cancer. Moreover, these are the first data to correlate the ratio of KLF6 wt/sp mRNA at the time of surgery for pancreatic cancer with reduced survival.

KLF6 immunostaining of pancreatic tissue identified specific cytoplasmic accumulation within tumor cells, similar to colorectal cancer (15). Although not clearly understood at the time of the earlier report (15), a likely mechanism appears to be the specific accumulation of KLF6 splice forms, which can accumulate in the cytoplasm because it lacks a nuclear localization signal. In pancreatic cancer, the tumor cells and microvessels exhibited KLF6 immunoreactivity, whereas no staining was observed in ductal cells of normal pancreas tissue. Western blot analysis of pancreatic cancer tissue using the same antibody used for immunohistochemistry confirmed the presence of lower MW KLF6 isoforms of ~40, ~30, and 26 kD. It is possible, but less likely, that the cytoplasmic accumulation represented full length KLF6, as this antibody does not distinguish between full length KLF6 and its splice forms. However, full length KLF6 typically appears in the nucleus in normal tissues but not in the cytoplasm (32).

While our study did not examine the biologic activity of KLF6 splice forms in normal pancreas and pancreatic cancer tissues, previous studies in prostate (20, 22) and ovarian (17) cancers clearly indicate a growth-promoting activity of the SV1 isoform. The mechanism of SV1's proliferative activity is not fully clarified, but likely reflects in part the sequestration of wild type, full length KLF6 protein in the cytoplasm (data not shown). Alternatively, a mechanism independent of direct KLF6 antagonism cannot be excluded. Regardless, the KLF6 SV1 isoform functionally antagonizes the ability of KLF6wt to suppress cell proliferation and tumorgenicity in vivo (20, 22). Increased alternative KLF6 splicing has an inhibitory effect on p21 and possibly other transcriptional targets (20, 22).

Two recent studies, one in primary lung cancer samples and the other in esophageal cancer cell lines have documented reduced expression of KLF6 mRNA as a result of gene silencing due to hypermethylation (33, 34). Furthermore, de novo KLF6 methylation may contribute to gene inactivation in astrocytic glioma, where mutations and LOH are shown to play only a minor role in KLF6 inactivation (35). Regardless of the mechanism, decreased expression of wild type KLF6 has been identified in other tumors by microarray analysis (36). However, the mechanisms underlying this reduction have not been elucidated, and the results were not validated by real time PCR.

In contrast, in the present study the reduced ratio of KLF6wt/sp mRNA in pancreatic tumor samples was primarily due to enhanced splice form expression rather than reduced KLF6 full length mRNA. It appears that net KLF6 activity is regulated in part by a critical balance between KLF6wt and alternatively KLF6 spliced forms (ratio mRNA KLF6wt/sp). However, it is not clear whether the biologic effects of this ratio are the same regardless of whether the ratio is altered as a result of an increase in splice form expression, or a reduction in full-length mRNA expression. Of great significance, however, an increased KLF6wt/sp ratio is associated with increased tumor differentiation. Moreover, the association of increased KLF6wt/sp ratio with survival in patients with pancreas cancer raises the possibility of using

this ratio as an independent predictor of prognosis. However, larger, prospective studies are required to establish such a role.

In conclusion, pancreatic cancer is associated with enhanced alternative splicing of the KLF6 tumor suppressor gene without associated LOH or gene mutation. Based on its close correlation with survival, the ratio of KLF6wt/sp mRNA is a potential prognostic marker whose value should be further validated in animal models and prospective human studies.

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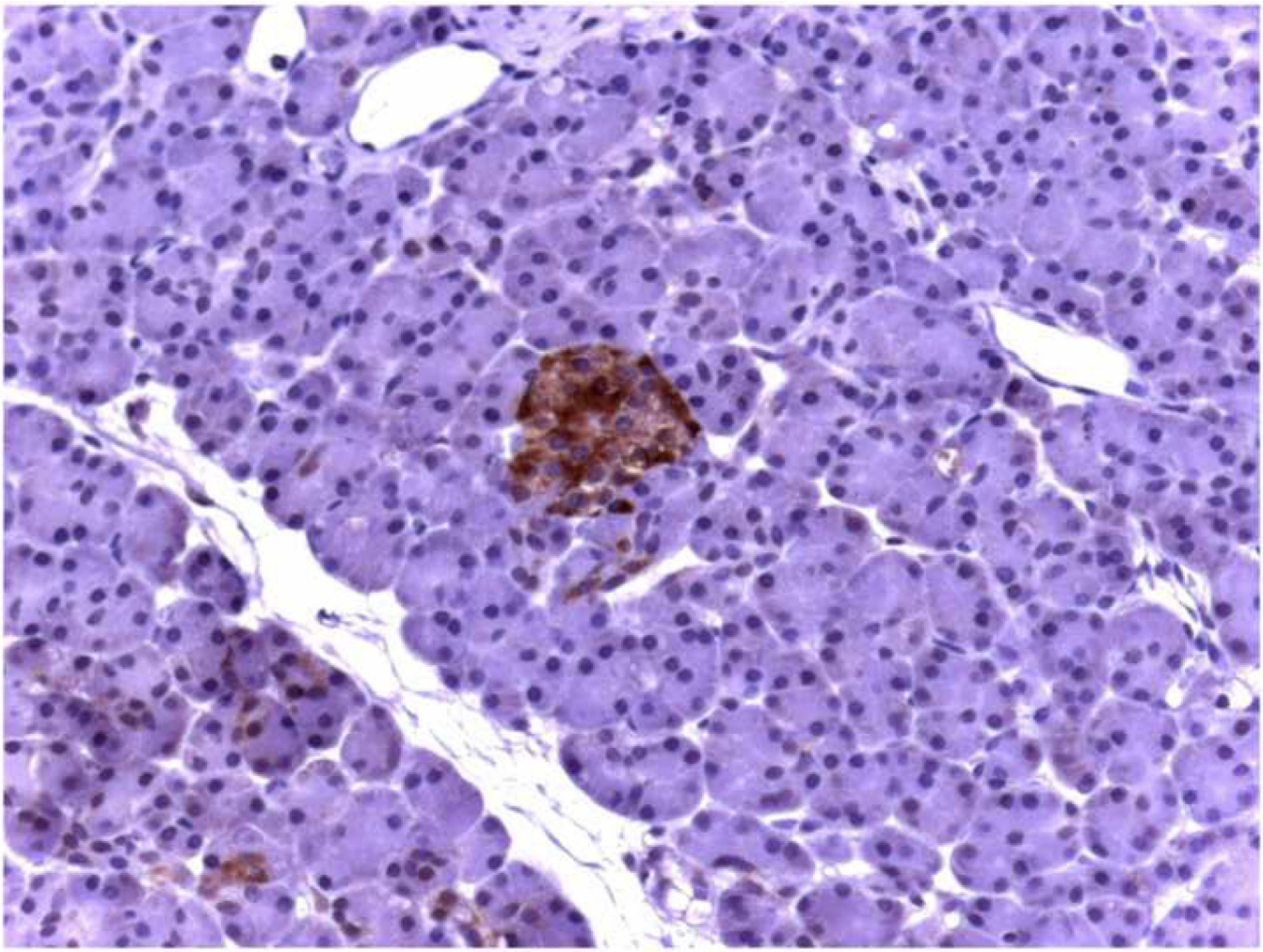

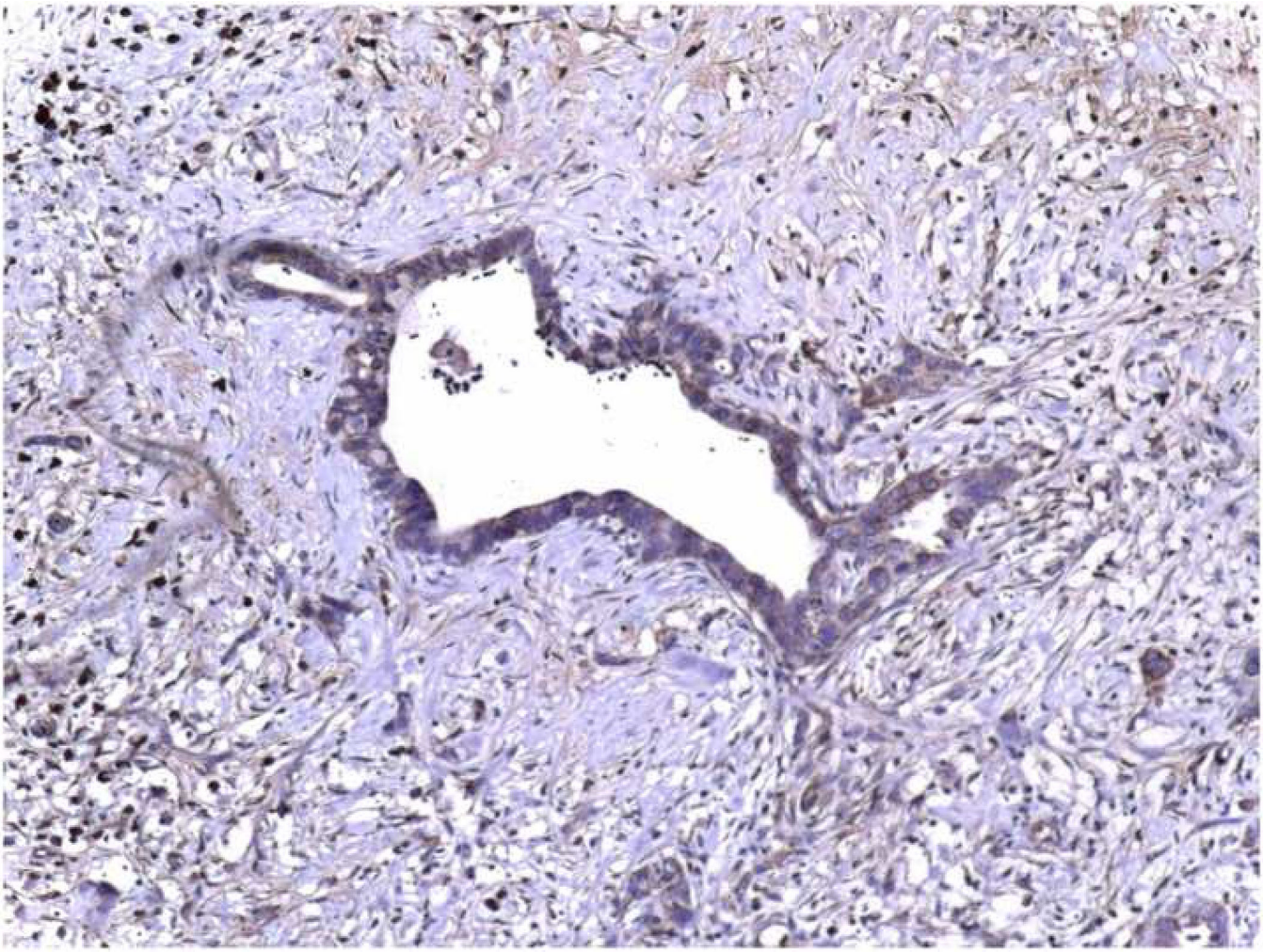

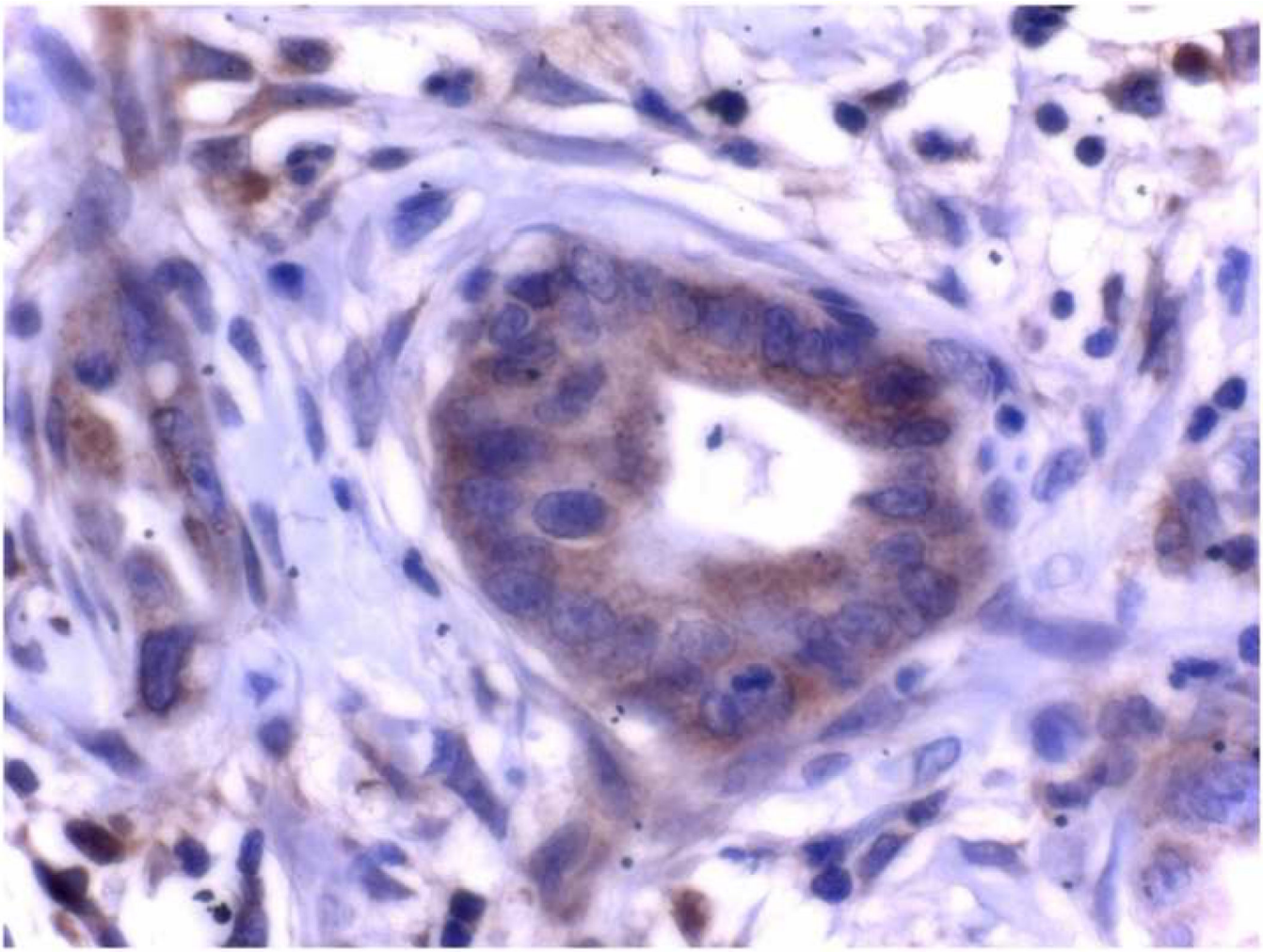

**Figure 1: Localization of KLF6 in normal human pancreatic tissue and pancreatic cancer samples**

(**A**) Normal pancreas, displays KLF6 immunostaining in scattered islet cells, faint staining in ductal cells, but no staining in acinus cells (× 50). (**B**) Ductal adenocarcinoma of the pancreas, demonstrating scattered cytoplasmatic KLF6 immunoreactivity, with some stromal staining. (× 50). **(C)** Higher power immunostaining for KLF6 in a separate pancreatic adenocarcinoma demonstrating the cytoplasmic and stromal staining (× 400). Insert showing no immunoreactivity of ductal cancer cells in control tissue.

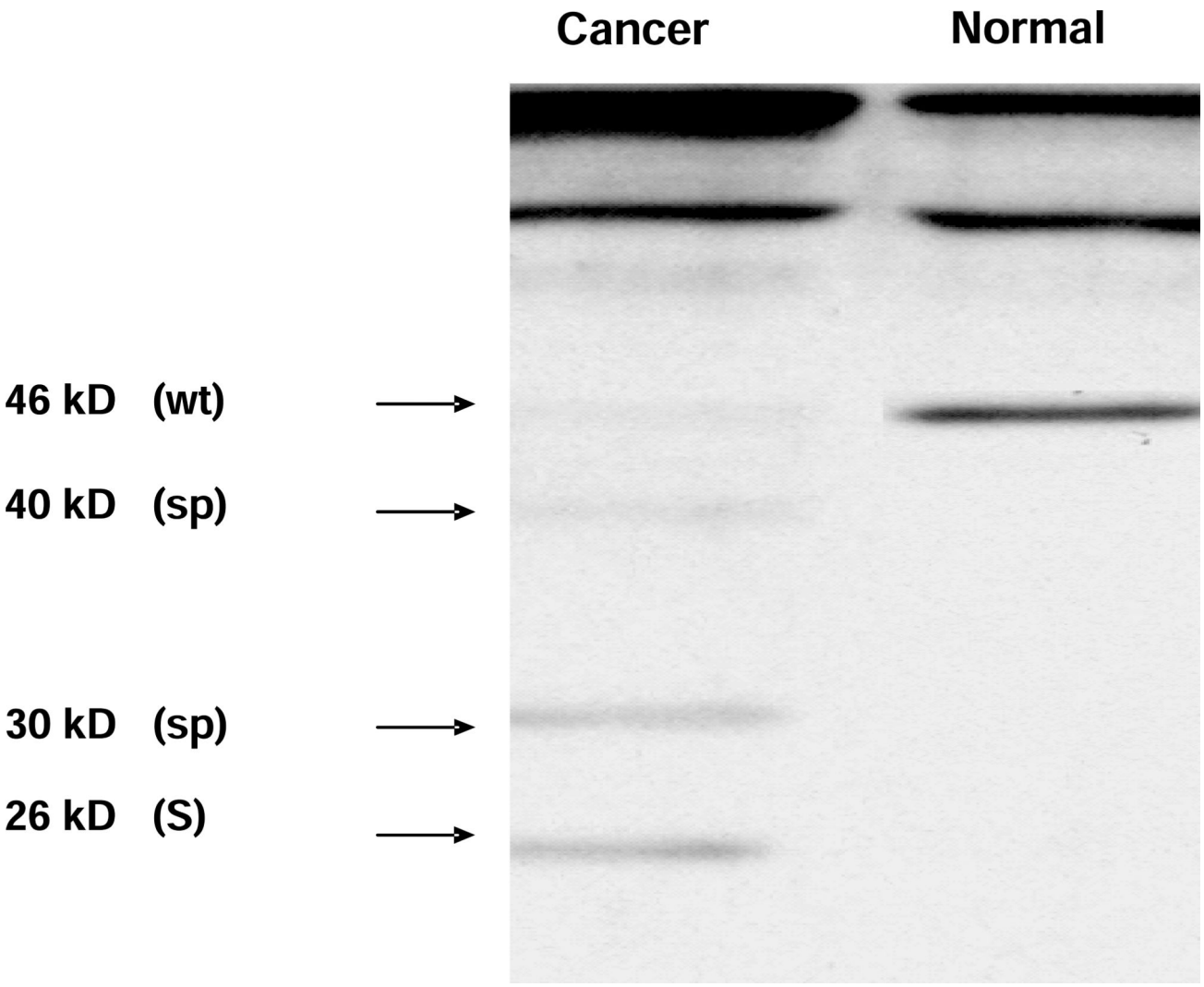


**Figure 2: Expression of KLF6wt protein and KLF6 splice forms in normal human pancreatic tissue and pancreatic cancer samples**

Representative Western blots are shown with pancreatic cancer at the left column and normal pancreas at the right column. Pancreatic cancer samples revealing a faint protein band at 46 kD representing KLFwt and bands at 26 kD, 30 kD, and 40 kD representing known forms of KLF6. In contrast, KLF6 protein expression in normal pancreas showing only one strong band at 46 kD representing KLF6wt.

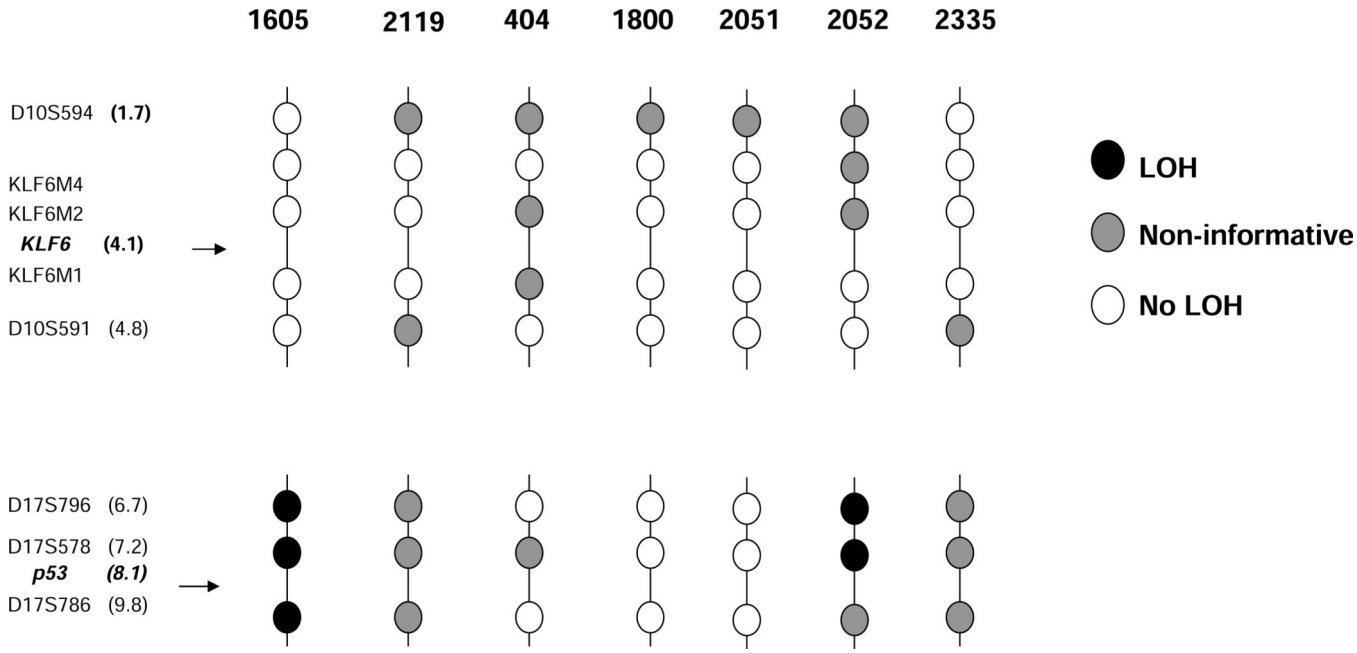


**Figure 3: Loss of heterozygosity (LOH) of the KLF6 and p53 genes in human pancreatic cancer samples**

LOH of the KLF6 and p53 locus was analyzed in tumor tissue from seven patients using microsatellite markers (vertical axis, with patient # above each axis) from the 10p15 region and KLF6-specific markers KLF6M1, M2 and M4. These markers flank the KLF6 gene by approximately 40 Kb centromerically, 10 Kb and 20 Kb telomerically. The lower half displays microsatellite markers flanking the p53 gene. Black filled circle - LOH; gray - non-informative (NI); white circle - no evidence of loss.

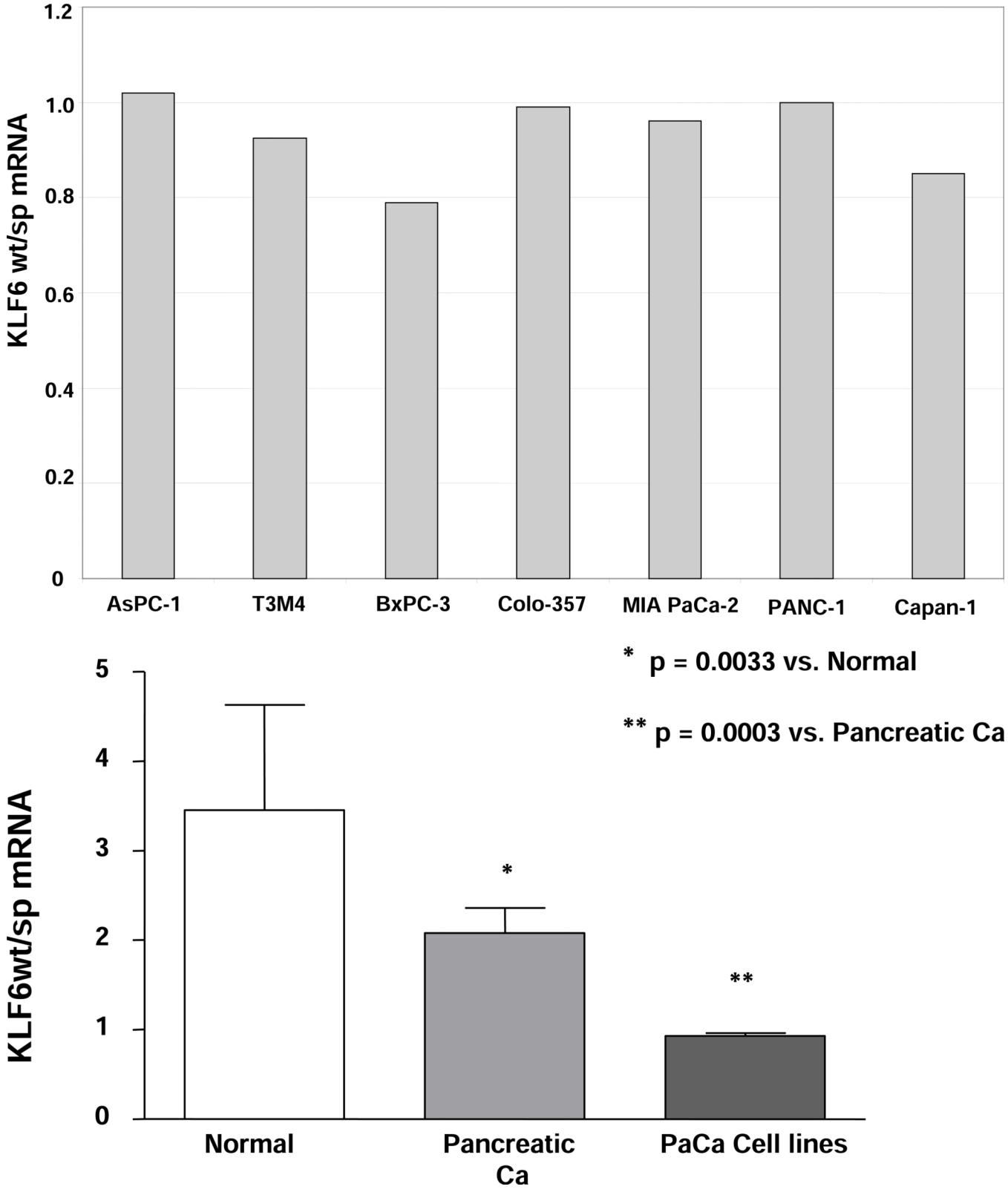


**Figure 4:**

(A) *Increased KLF6wt/sp mRNA ratio in human pancreatic cancer cell lines*

Quantitative real-time PCR of extracted total RNA from the seven human pancreatic cancer cell lines was performed as described in Materials and Methods. KLF6sp expression was calculated by determining the difference between KLF6 total mRNA and KLF6wt mRNA alone. All analyses were performed in triplicate and normalized to GAPDH mRNA expression, and values are markedly reduced compared to normal pancreatic tissue and primary pancreatic cancers (see Figure 4B).

(B): *Decreasing ratio of KLF6wt/sp mRNA in pancreatic cancer and pancreatic cancer cell lines.*

The mean ratio of KLF6wt/sp mRNA of 24 human pancreatic cancer tissues compared to specimens of normal human pancreas from 8 individuals. These data are compared to the median ratio of KLF6wt/sp mRNA from the 7 human pancreatic cancer cell lines in panel A. Error bars represent the SEM of three different experiments. Statistical analysis revealed a significantly higher ratio in normal pancreatic tissues versus pancreatic cancer tissues ($p=0.0033$) and pancreatic cancer tissues versus pancreatic cancer cell lines ($p=0.0003$), respectively.

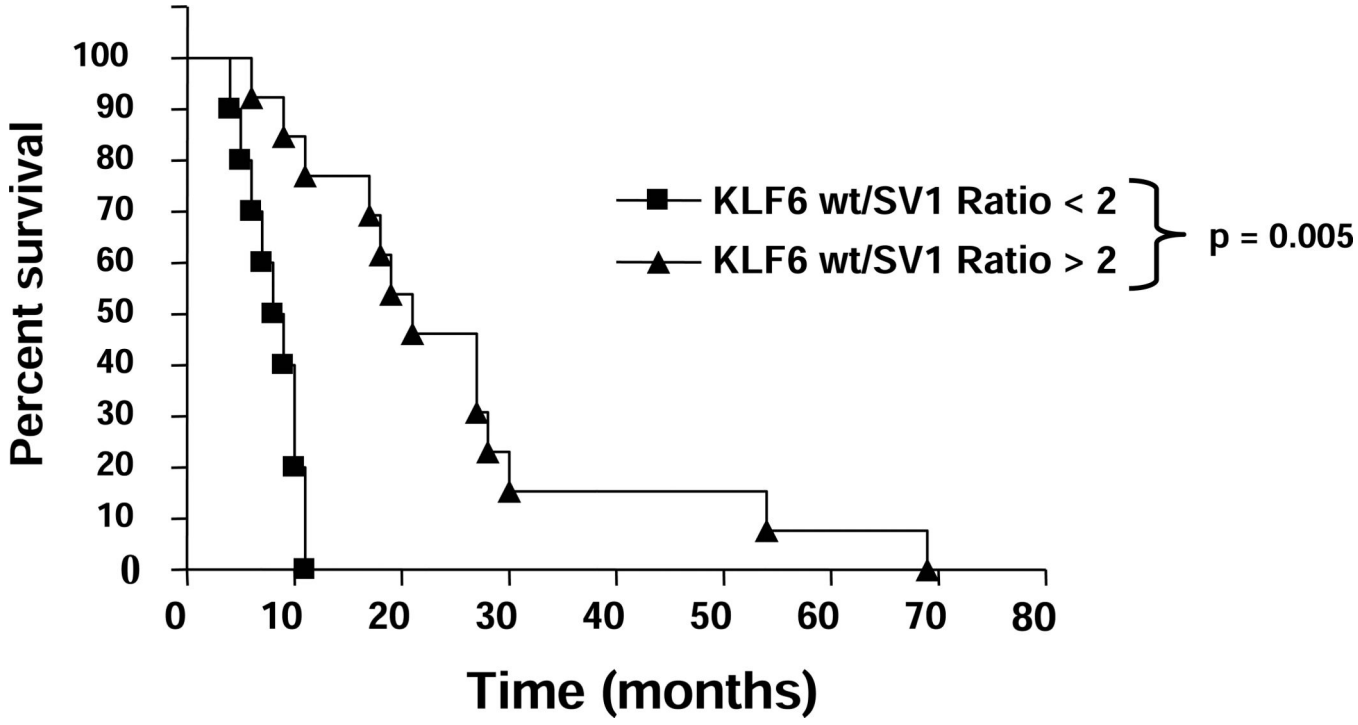


**Figure 5: The ratio of KLF6wt/sp mRNA expression ratio correlates with survival in patients with pancreatic cancer.**

The KLF6wt/sp ratio was determined from mRNA that was extracted from tumors harvested immediately following resection in patients with proven ductal adenocarcinoma of the pancreas. The ratio was significantly correlated with overall survival in these patients. The median survival of patients with KLF6wt/sp ratio >2 (n=14) was 21 months, which was significantly longer than the median survival of 9 month in patients (n=10) with KLF6wt/sp ratio ≤ 2 (*p=0.005*).

**Table 1:**

**Clinical data**

Clinical data and mRNA KLF6wt/sp in patients with pancreatic carcinoma (n=24) and normal pancreas* (n=8). G = grading; pT, pN, pM according to the TNM system; R = classification of residual tumor.

| Patient | Gender | Age | G | UICC | pT | pN | pM | R | Survival [months] | KLF6wt/sp mRNA |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 68 | 3 | II | 3 | 0 | 0 | 0 | 10 | 1.85 |
| 2 | M | 69 | 3 | II | 3 | 0 | 0 | 0 | 7 | 1.75 |
| 3 | M | 66 | 3 | III | 4 | 1 | 0 | 0 | 10 | 1.85 |
| 4 | M | 53 | 1 | I | 1 | 0 | 0 | 0 | 28 | 2.33 |
| 5 | W | 66 | 2 | III | 3 | 1 | 0 | 0 | 69 | 2.63 |
| 6 | M | 70 | 2 | III | 3 | 1 | 0 | 0 | 6 | 2.04 |
| 7 | W | 77 | 2 | III | 4 | 1 | 0 | 0 | 4 | 1.32 |
| 8 | M | 75 | 1 | I | 2 | 0 | 0 | 0 | 54 | 3.57 |
| 9 | M | 61 | 3 | II | 4 | 0 | 0 | 0 | 18 | 2.38 |
| 10 | W | 71 | 2 | IV | 4 | 1 | 1 | 0 | 11 | 2.27 |
| 11 | W | 60 | 2 | III | 3 | 1 | 0 | 0 | 9 | 2.27 |
| 12 | W | 52 | 3 | III | 4 | 1 | 0 | 0 | 5 | 1.75 |
| 13 | M | 72 | 1 | III | 2 | 1 | 0 | 0 | 30 | 2.63 |
| 14 | M | 75 | 1 | II | 3 | 0 | 0 | 0 | - | 2.17 |
| 15 | W | 52 | 1 | I | 2 | 0 | 0 | 0 | 19 | 2.13 |
| 16 | M | 76 | 2 | III | 2 | 1 | 0 | 0 | 17 | 2.08 |
| 17 | W | 83 | 1 | I | 2 | 0 | 0 | 0 | 27 | 3.57 |
| 18 | W | 73 | 2 | III | 4 | 1 | 0 | 0 | 27 | 2.50 |
| 19 | W | 52 | 3 | III | 2 | 1 | 0 | 0 | 8 | 0.48 |
| 20 | W | 56 | 2 | III | 2 | 1 | 0 | 0 | 11 | 1.52 |
| 21 | W | 64 | 1 | II | 3 | 0 | 0 | 0 | 21 | 2.08 |
| 22 | M | 57 | 3 | III | 3 | 1 | 0 | 0 | 11 | 1.59 |
| 23 | W | 69 | 3 | III | 3 | 1 | 0 | 0 | 9 | 1.82 |
| 24 | W | 81 | 3 | III | 3 | 1 | 0 | 0 | 6 | 1.39 |
| 25* | W | 50 | | | | | | | | 5.50 |
| 26* | M | 38 | | | | | | | | 1.61 |
| 27* | M | 43 | | | | | | | | 3.45 |
| 28* | M | 25 | | | | | | | | 4.00 |
| 29* | F | 45 | | | | | | | | 2.94 |
| 30* | F | 50 | | | | | | | | 3.45 |
| 31* | M | 39 | | | | | | | | 5.26 |
| 32* | M | 41 | | | | | | | | 2.63 |

**Table 2:**
**Correlation of clinical data with mRNA KLF6wt/sp**

Clinical data and ratio of mRNA KLFwt/sp expression were correlated and statistical analysis was performed using the Spearman test. Additionally, Mann Whitney Test was used to analyze the differences between KLF6 ratio of normal patients and cancer patients.

| Characteristics | Normal pancreas KLF6wt/sp (avg) | Pancreas cancer KLF6wt/sp (avg) | P-value |
|---|---|---|---|
| **Patients (n)** | 3.45(8) | 2.08 (24) | 0.002 |
| **Grading** | | | 0.001 |
| **1 (n)** | - | 2.33 (7) | |
| **2 (n)** | - | 2.18 (8) | |
| **3 (n)** | - | 1.75 (9) | |
| **Tumor stage** | | | 0.076 |
| **I (n)** | - | 2.95 (4) | |
| **II (n)** | - | 2.08 (5) | |
| **III (n)** | - | 1.84 (14) | |
| **IV (n)** | | 1.5 (1) | |
| **Survival** | | | 0.0001 |