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

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ABSTRACT

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

Whereas neither KLF6 loss nor mutation was identified, expression of the KLF6 alternative splice forms was significantly increased in pancreatic tumour 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 localisation signal. In addition, KLF6 splicing correlated significantly with tumour 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.

1. Introduction

Krüppel-like factor 6 (KLF6) is a member of the Krüppel-like factor family of transcription factors; this family contains at least 20 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 characterisation as a growth suppressor, led to the identification of KLF6 as a tumour suppressor gene frequently inactivated in prostate cancer.[8] Growth suppressive mechanisms of KLF6 include transcriptional induction of p21 in a p53-independent manner8, upregulation of TGFβ1 and its receptors9, inactivation of c-jun, [10] inhibition of other proto-oncogene signalling 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

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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 tumour xenografts, thereby confirming their growth-promoting, tumourigenic 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 amongst the most lethal of solid organ tumours. 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 characterised 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 end-points.

2. Materials and methods

2.1. *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 tumours, five with stage II, 14 with stage III and one with stage IV tumours. Tumour 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 (ranges: 8–27) (Table 2). The study was approved by the Ethics Committee of the University of Berne, Switzerland, and the University of Heidelberg, Germany.

2.2. *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 foetal 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).

2.3. *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 deparaffinised 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 recognising 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 non-specific IgG antibody; immunostaining was not detected in either case. All slides were analysed by two independent observers blinded to patient status. Any differences in the findings were resolved by joint review and consultation with a third observer.

2.4. *Quantitative PCR for KLF6wt and KLF6sp in human pancreatic cancer tissue and cultered pancreas tumour cells*

For quantifying target gene expression, RNA isolation from cultured cells and patient samples was performed using

Table 1 – Clinical data

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

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

Table 2 – Correlation of clinical data with KLF6wt/sp mRNA

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]	
Tumour 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

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 analyse the differences between KLF6 ratio of normal patients and cancer patients.

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 optimised 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) was 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 normalised 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.

2.5. 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 labelled 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 and 38 cycles. The data were analysed 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.

2.6. Western Blot

Pancreatic cancer cell lines ($n = 7$), human pancreatic cancer tissues ($n = 7$) and human normal pancreas tissue ($n = 5$) were homogenised 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 homogenised material was collected and centrifuged at 4 °C for 30 min at 14,000*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. Visualisation was performed by the enhanced chemoluminescent method (Amersham International, Freiburg, Germany).

2.7. Statistical analysis

The age and survival of all patients are reported as median and range. Furthermore, we analysed the median KLF6wt/sp ratio according to the four tumour stages (UICC) and tumour grades (I, II, III).[27] Differences between the groups were analysed 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 tumour grading, tumour stage and survival was analysed by the Spearmans test. Bivariate analysis was done by Mann–Whitney test.

3. Results

3.1. 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 (Fig. 1A).

Ductal adenocarcinoma of the pancreas: In contrast to normal pancreas, ductal carcinoma cells displayed marked KLF6 immunoreactivity within the cytoplasm of tumour cells. Acinar cells and normal ducts within the cancer tissue were negative, and only some micro-vessels surrounding the tumour 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 amongst 15 samples analysed, and a representative photomicrograph is shown (Fig. 1B and C).

3.2. KLF6. protein isoforms in normal pancreas and pancreatic carcinoma

Given the marked over-expression of cytoplasmic KLF6 in pancreatic tumour samples we sought to specifically define which KLF6 isoforms were present in both normal and cancerous pancreatic tissues. 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

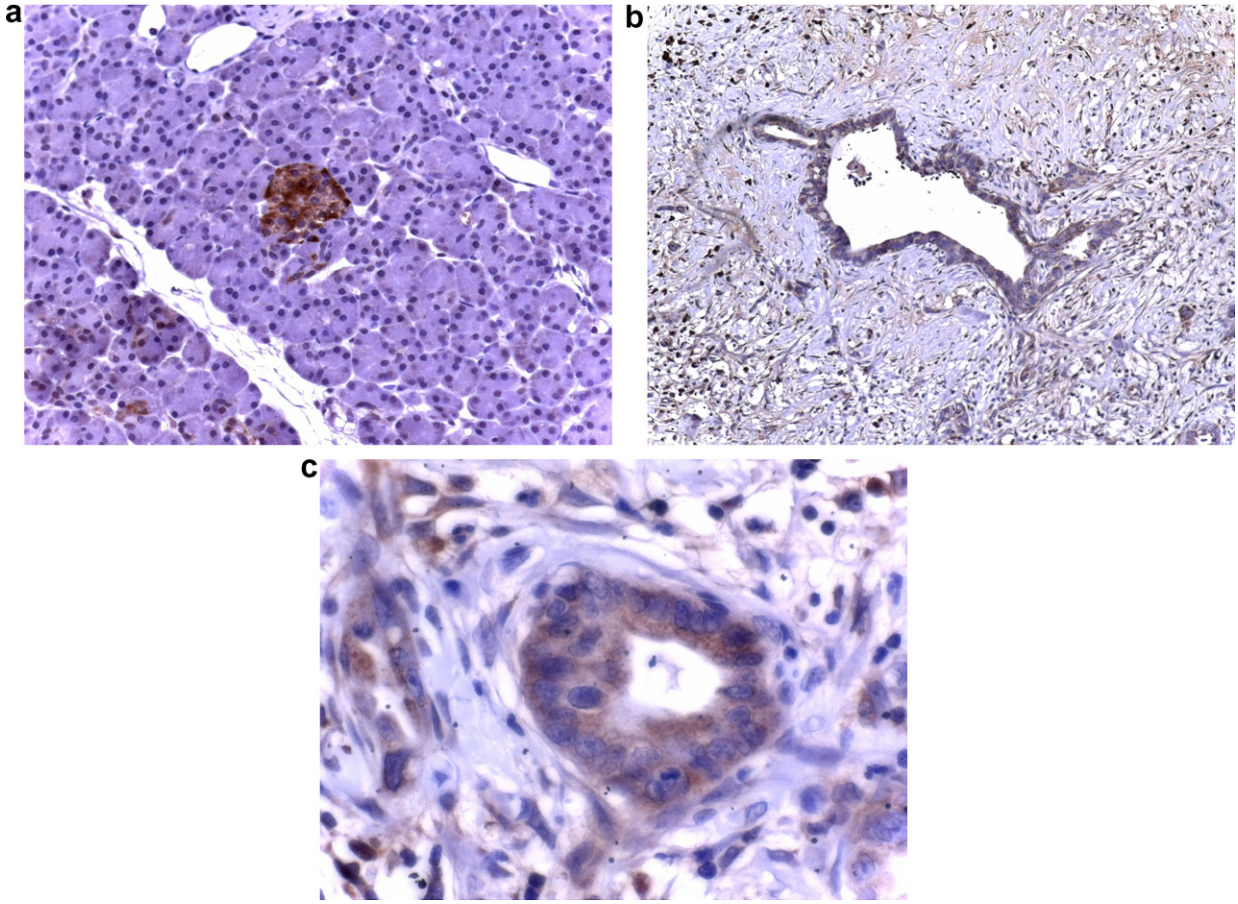

Fig. 1 – Localisation of KLF6 in normal human pancreatic tissue and pancreatic cancer samples: (a) Normal pancreas displays KLF6 immunostaining in scattered islet cells, faint staining in ductal cells, but no staining in acinus cells (×50). (b) Ductal adenocarcinoma of the pancreas, demonstrating scattered 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.

KLF6. These lower MW bands are consistent with the previously described alternatively spliced products of KLF6 [20] (Fig. 2).

3.3. *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 tumour 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 analysed 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 (Fig. 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 tumour suppressor gene alterations is common.[31]

3.4. *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 was analysed 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 tumours (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 analysed 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 (Fig. 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; Fig. 4). These data

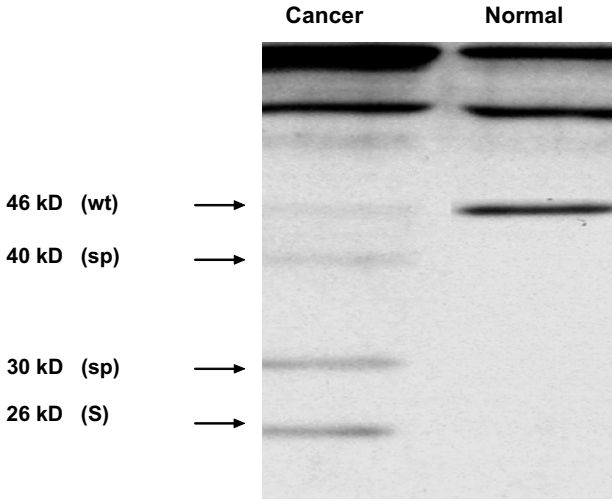

Fig. 2 – Expression of KLF6wt protein and KLF6 splice forms in normal human pancreatic tissue and pancreatic cancer samples. Representative Western blots are shown with pancreatic cancer at the left column and normal pancreas at the right column. Pancreatic cancer samples revealing a faint protein band 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.

indicate that KLF6 alternative splicing is increased in pancreatic cancer cell lines even more than in primary pancreatic cancers.

3.5. *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 tumour 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 tumours, (G2), and 1.75 (range: 1.49–1.83) in poorly differentiated cancer samples (G3). KLF6wt/sp was significantly related to the tumour grade (G1–G3), with more poorly differentiated tumours having a lower KLF6wt/sp ratio than well-differentiated tumours ($p = 0.001$; 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 tumour 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 analysis 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; $p = 0.005$) (Fig. 5). Furthermore, the Cox-regression revealed KLF6 ratio as an independent marker for survival ($p = 0.006$).

4. Discussion

In this study, we have characterised the KLF6 allele status and the expression of KLF6 mRNA in human pancreatic tumours

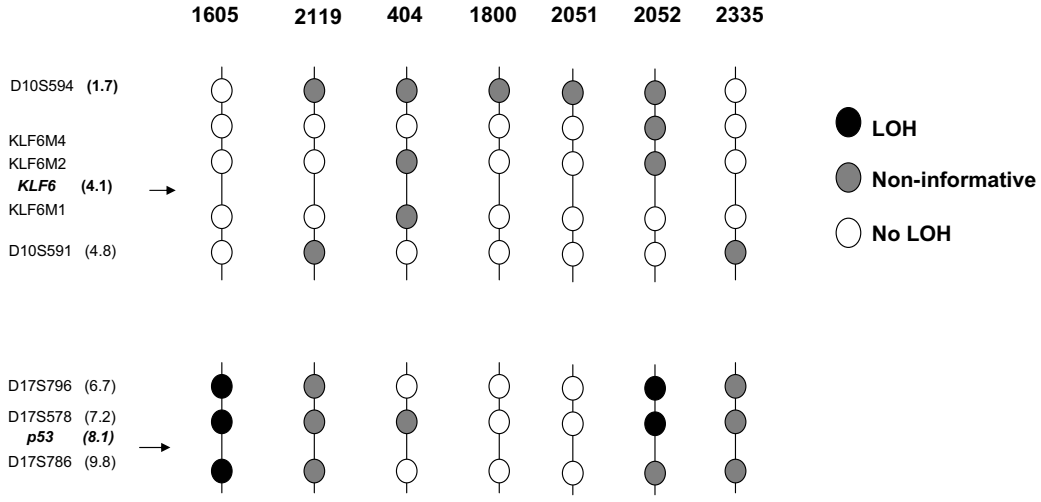

Fig. 3 – Loss of heterozygosity (LOH) of the KLF6 and p53 genes in human pancreatic cancer samples. LOH of the KLF6 and p53 locus was analysed in tumour tissue from seven patients using microsatellite markers (vertical axis, with patient # above each axis) from the 10p15 region and KLF6-specific markers 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.

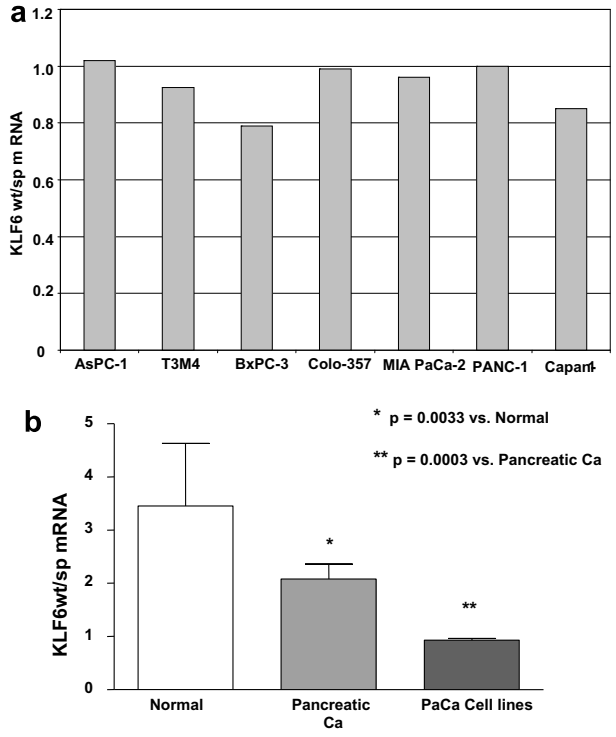

Fig. 4 – (a) Increased KLF6wt/sp mRNA ratio in human pancreatic cancer cell lines. Quantitative real-time PCR of extracted total RNA from the seven human pancreatic cancer cell lines was performed as described in 'Materials and methods'. KLF6sp expression was calculated by determining the difference between KLF6 total mRNA and KLF6wt mRNA alone. All analyses were performed in triplicate and normalised to GAPDH mRNA expression, and values are markedly reduced compared to that of normal pancreatic tissue and primary pancreatic cancers (see b). (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 that of specimens of normal human pancreas from 8 individuals. These data are compared to the median ratio of KLF6wt/sp mRNA from the seven 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.

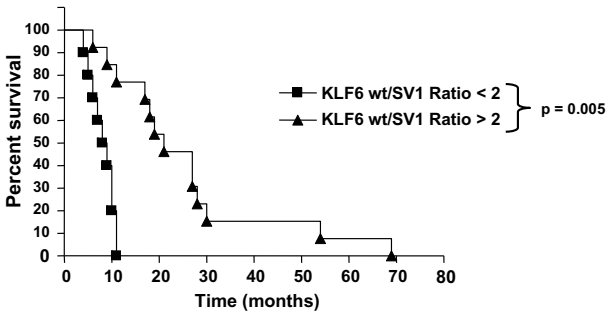

Fig. 5 – The ratio of KLF6wt/sp mRNA expression ratio correlates with survival in patients with pancreatic cancer. The KLF6wt/sp ratio was determined from mRNA that was extracted from tumours harvested immediately following resection in patients with proven ductal adenocarcinoma of the pancreas. The 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 $\leqslant 2$ ($p = 0.005$).

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-characterised set of tumours 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 tumour 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 localisation signal. In pancreatic cancer, the tumour 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]

Whilst 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 antagonises the ability of KLF6wt to suppress cell proliferation and tumourgenicity *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 oesophageal 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 tumours 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 this study the reduced ratio of KLF6wt/sp mRNA in pancreatic tumour 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 tumour 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 tumour 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.

Conflict of interest statement

None declared.

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