Tumorigenesis and Neoplastic Progression

KLF4 is a Novel Candidate Tumor Suppressor Gene in Pancreatic Ductal Carcinoma

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Ductal pancreatic carcinoma (DPC) is a deadly disease with an incidence of 9 cases in 100,000 people per year and a mortality rate close to 100%. Allelic losses in the long arm of chromosome 9 are commonly encountered in many human malignancies but no data are yet available about DPC. We screened 40 laser-microdissected DPC samples and 6 pre-invasive lesions for 9 microsatellite mapping markers of region 9q21.3 through 9q34.2. A small overlapping region of deletion, spanning 8 million base pairs, was identified between D9S127 and D9S105. Two genes, RSG3 and KLF4, mapped to 9q31.1 through 9q32, were further investigated. A highly significant association was found between KLF4 gene expression levels and genomic status. Similarly, absence of immunohistochemical expression of KLF4 protein was found in 86.8% cases of DPC (33/38). Overexpression of KLF4 in a human pancreatic carcinoma cell line induced a significant decrease in the proliferation associated with up-regulation of p21 and the down-regulation of cyclin D1. In conclusion, we identified a novel oncosuppressor region located at the 9q31.1-3 locus that is lost in DPC at high frequency. Loss of KLF4 expression is closely related to the genomic loss, and its restoration inhibits cancer cell proliferation, suggesting a key suppressor role in pancreatic tumorigenesis. (*Am J Pathol* 2011, 178:361–372; DOI: 10.1016/j.ajpath.2010.11.021)

Pancreatic cancer is the fifth leading cause of cancer-related death in both men and women in the Western world, being responsible for 5% of all cancer-related deaths.¹ The lack of reliable early diagnostic methods and effective therapeutic regimens makes the mortality rates in persons with pancreatic carcinoma virtually the same as the incidence rates. A radical surgical approach is possible in only 10% of cases, and adjuvant therapies are virtually ineffective.² A better understanding of the molecular mechanisms leading to pancreatic tumorigenesis may provide new markers for early diagnosis and potential targets for therapeutic intervention.

Ductal pancreatic carcinoma (DPC) is by far the most common pancreatic tumor type, accounting for about 90% of all pancreatic malignancies. At present, a molecular model of DPC development has been proposed; it involves key genes, such K-ras, HER2neu, p16, p53, and DPC4 (smad4). Activating mutations in the K-ras oncogene and the overexpression of Her-2/neu gene are considered “early” genetic events because they occur in pre-invasive lesions (pancreatic intra-epithelial neoplasias, or PanINs)^{3,4} and are followed by homozygous deletions in the p16 tumor suppressor gene locus.^{5,6} Later in the tumor’s progression, inactivations of p53 and DPC4 tumor suppressor genes are thought to be key events that lead to fully transformed phenotypes (carcinoma *in situ*, or PanIN-3).^{7–11}

Wide genomic instability characterizes DPC, as has been documented by the large collection of cytogenetic abnormalities reported in the literature.^{12–17} Among these, 9p (p16 locus), 17p (p53 locus), and 18q (DPC4 locus) have been reported to have been deleted in more than 60% of cases, followed by the deletions of 3p, 4q, 6q, 8p, 10q, 12q, 13q, 21q, and

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22q, which have been found to have been deleted in 40% to 60% of cases.¹⁸ Allelic losses in the long arm of chromosome 9, however, have never been described in pancreatic cancer, although they are commonly encountered in many other human malignancies, including bladder, gastric, colorectal, and esophageal carcinomas.^{19–23} In particular, loss of heterozygosity (LOH) analysis of 9q at 9q13-31 and 9q34 is the most common genetic abnormality in bladder cancer, being present in 56% to 71% of cases¹⁹ and is often observed in 25% to 50% of sporadic colorectal cancers²¹ and in 56.5% of gastric cancers.²⁰ These data strongly suggest the presence of a tumor suppressor gene in this area. In fact, two very interesting genes, KLF4 and RGS3, map to this region and have been implicated in cancer development: KLF4 maps exactly to position 9q31.2, and its expression is associated with growth arrest.^{24,25} It has been reported to be an essential mediator of p53 in preventing cell cycle progression following DNA damage,^{26,27} and KLF4 has been described as a tumor suppressor gene in colorectal,²⁸ bladder, and gastric cancers.^{29–31}

The RGS3 gene maps to the same area and behaves as an important negative regulator of G protein-coupled mitogenic signal transduction and as an activator of the mitogen-activated protein kinase signaling cascade, which is implicated in cellular proliferation, transformation, and oncogenesis. Several mammalian regulators of G protein signaling (RGSs), including RGS1, RGS2, RGS3, and RGS16, have been shown to be regulated transcriptionally by mitogens, serum, inhibition of protein synthesis, and activation of p53 tumor suppressor.^{32–34} These findings led us to investigate, through LOH analysis, how this region of chromosome 9q in DPC is involved in both infiltrative tumors and PanINs. We further studied the expression levels of both the KLF4 and RGS3 genes in relation to the structural status of the long arm of chromosome 9. The finding that there was an important involvement of the KLF4 gene in DPC led us to proceed with further molecular and functional analyses, which identified a novel and early role for KLF4 in pancreatic tumorigenesis. The dramatically negative prognosis for patients affected by pancreatic cancer inspires the development of new molecular therapeutic strategies that must be based on detailed knowledge of the molecular pathways of the disease.

Materials and Methods

The potential suppressor role in human DPC cancer tissues of the KLF4 and RGS3 genes was studied through 9q LOH analysis and mutational, methylation, and expression studies. KLF4 protein expression was measured by immunohistochemistry (IHC). Finally, KLF4 functional studies were conducted on PANC-1. Laser microdissection was used to select the neoplastic cell populations from histological sections.

Tissues

Samples of DPC and pre-invasive lesions (PanIN-1A and PanIN-2) were collected at the Division of Surgical, Molecular, and Ultrastructural Pathology at the University Hospital of Pisa (Table 1).

For expression experiments, reference normal samples were collected from explanted pancrea.

9q Loss of Heterozygosity Analysis

We used 40 microdissected, fresh DPC DNAs and matched blood samples. Tissues and blood were quickly frozen in liquid nitrogen and stored at -80°C . We obtained 6 microdissected pre-invasive lesions (four PanIN-1A and two PanIN-2) from formalin-fixed and paraffin-embedded pancreatic fragments of DPC cases (Table 1). They were analyzed together with their matched blood samples.

KLF4 and RGS3 Gene Expression Analysis

We extracted mRNA from 35 and 33 DPCs, respectively, for which LOH data were available too (Table 1). We used 6 microdissected, freshly frozen normal pancreatic ducts for comparison analysis of KLF4 gene expression.

KLF4 Mutational Analysis

We used 17 DPC samples with available LOH data.

KLF4 Promoter Methylation Status

We used 12 DPC samples that had available LOH data. PANC 1 cell lines were also analyzed.

KLF4 Protein Expression

IHC was performed on a total of 38 DPC samples, 17 of which had expression data available. We also analyzed 6 pre-invasive lesions (Table 1).

KLF4 Functional Studies

PANC-1 and BxPC3 cell lines originating from human pancreatic cancer cells were used.

Laser Microdissection

Histological sections 6 μm thick were transferred to microscope glass slides coated with a membrane for microdissection (two to three sections per slide). Slides were stained using H&E RNAase-free products. Sections were dehydrated (ethanol 100% for 30') and immersed in xylene for 1 minute to facilitate their separation during the microdissection. The xylene was allowed to evaporate completely from the sections at room temperature. Microdissection was performed under direct microscopic visualization ($\times 10$) using the Leica AS LDM system (Houston, TX). About 5000 microdissected cells were isolated and placed in 20 μL of RNA extraction mix.

Table 1. Samples Analyzed in the Study*

Tissue sample	TNM class	Grading	DNA-LMD	RNA-LMD	LOH in the SOR	KLF4 mRNA expr.	RGS3 mRNA expr.	IHC KLF4
3-DPC	pT4 No Mx	G3/3	*	*	<i>HomDeL</i>	—	+	neg
1-DPC	pT3 N1b M1	G2/3	*	*	<i>HomDeL</i>	—	+	
2-DPC	pT3 N0 Mx	G3/3	*	*	<i>HomDeL</i>	—	+	
23-DPC	pT3 N0 Mx	G3/3	*	*	<i>HomDeL</i>	—	—	
27-DPC	pT3 N1 M1	G2/3	*	*	<i>HomDeL</i>	—	+	
13-DPC	pT3 N1 Mx	G2/3	*	*	LOH	—	/	neg
28-DPC	pT3 N1 Mx	G2/3	*	*	LOH	—	+	neg
42-DPC	pT3 N1 Mx	G3/3	*	*	LOH	—	+	neg
44-DPC	pT3 N1 Mx	G2/3	*	*	LOH	—	+	neg
61-DPC	pT3 N0 Mx	G2/3	*	*	LOH	—	+	neg
66-DPC	pT3 N1 Mx	G3/3	*	*	LOH	—	+	neg
67-DPC	pT3 N1 Mx	G3/3	*	*	LOH	—	—	neg
19-DPC	pT3 N1b Mx	G3/3	*	*	LOH	—	+	
47-DPC	pT3 N1b Mx	G2/3	*	*	LOH	—	+	
53-DPC	pT3 N1b Mx	G1/3	*	*	LOH	—	+	
69-DPC	pT3 N1 Mx	G2/3	*	*	LOH	—	—	
74-DPC	pT3 N0 Mx	G3/3	*	*	LOH	—	—	
78-DPC	pT3 N1 M1	G2/3	*	*	LOH	—	—	
7-DPC	pT4 N1 Mx	G3/3	*	*	LOH	+	+	neg
30-DPC	pT4 N1a Mx	G2/3	*	*	LOH	+	+	
17-DPC	pT3 N1 Mx	G2/3	*	*	ROH	—	+	neg
43-DPC	pT3 N1 Mx	G2/3	*	*	ROH	—	+	neg
59-DPC	pT3 N1 M1	G2/3	*	*	ROH	—	+	neg
35-DPC	pT4 N1b Mx	G3/3	*	*	ROH	—	+	
40-DPC	pT3 N1b Mx	G2/3	*	*	ROH	—	—	
70-DPC	pT3 N1 Mx	G2/3	*	*	ROH	—	+	
76-DPC	pT3 N1 Mx	G3/3	*	*	ROH	—	—	
22-DPC	pT3 N1b Mx	G2/3	*	*	ROH	+	+	neg
29-DPC	pT3 N1 M1	G2/3	*	*	ROH	+	/	neg
24-DPC	pT3 N1 Mx	G3/3	*	*	ROH	+	—	pos
26-DPC	pT3 N0 Mx	G1/3	*	*	ROH	+	+	pos
33-DPC	pT3 N0 Mx	G3/3	*	*	ROH	+	+	pos
38-DPC	pT3 N1 Mx	G2/3	*	*	ROH	+	+	
57-DPC	pT3 N1 Mx	G2/3	*	*	ROH	+	+	
75-DPC	pT3 N1 Mx	G3/3	*	*	ROH	+	+	
Sample 1	PanIN	2	*		LOH			neg
Sample 2	PanIN	1A	*		<i>HomDeL</i>			neg
Sample 3	PanIN	1A	*		<i>HomDeL</i>			neg
Sample 4	PanIN	2	*		LOH			pos
Sample 5	PanIN	1A	*		LOH			pos
Sample 6	PanIN	1A	*		LOH			pos
6-DPC	pT3 N1b Mx	G2/3	*		LOH			
8-DPC	pT4 N1 Mx	G3/3	*		LOH			
73-DPC	pT3 N1b Mx	G3/3	*		LOH			
85-DPC	pT3 N1 Mx	G2/3	*		LOH			
77-DPC	pT3 N0 Mx	G1/3	*		ROH			
DPC1	pT3 N0 Mx	G2/3	/	/	/	/	/	pos
DPC2	pT3 N1 Mx	G2/3	/	/	/	/	/	pos
DPC3 to 21	various	various	/	/	/	/	/	neg

*DNA/RNA was extracted from laser microdissected (LMD) neoplastic cells (frozen tissue). Six PanINs were paraffin embedded; 21 paraffin-embedded DPCs were further analyzed only for KLF4 protein expression by IHC: 2 were KLF4-positive (DPC1,2), and 19 were KLF4-negative (DPC3-21).

Extraction of Nucleic Acids

Microdissected tumor cells were resuspended in 20 μ L of DNA extraction buffer containing 0.005 mol/L of Tris-HCl, 0.001 mol/L of EDTA, 1% of Tween-20, and 0.1 mg/ml of proteinase K (pH 8.0) and were incubated overnight at 37°C. After proteinase K inactivation at 95°C for 10 minutes, DNA samples were ready for amplification. RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

Analysis of Loss of Heterozygosity

We used a panel of nine highly polymorphic microsatellite markers for deletion mapping of the chromosome 9 long arm. Details about the cytogenetic localization of the microsatellite markers used are shown in Table 2. Primer information was obtained from the National Center for Biotechnology Information Genome Database (<http://www.ncbi.nlm.nih.gov/mapview/>) and Ensembl Database (<http://www.ensembl.org>). The region framed by the D9S152 and D9S66 markers spans approximately 50

Table 2. Frequency of LOH on 9q in DPC and in PanINs

Marker name	Mapping position	Genetic position (Mpb)*	N°LOH/informative DPCs	N°LOH/informative PanINs	LOH (%) DPCs	LOH (%) PanINs
D9S152	9q21.32	83,36	13/37	0/5	35	0
D9S127	9q31.1	103,6	16/38	2/4	42	50
D9S53	9q31.1	104,6	13/34	1/4	38	25
D9S105	9q31.3	111,3	19/37	5/6	51	83
D9S106	9q32	113,3	12/36	1/5	33	20
D9S1823	9q33.2	120,7	6/27	0/2	22	0
D9S125	9q34.13	132,1	8/31	3/6	26	50
D9S1199	9q34.13	132,8	10/37	1/5	27	20
D9S66	9q34.2	133,7	8/32	0/3	25	0

*Ensembl DataBank.

million base pairs. PCR reactions were performed on a final volume of 30 μ L containing 2 μ L of DNA lisate, 2 mmol/L deoxynucleotide triphosphate (Eurobio, Les Ulis, France), 250 ng/ μ L of each forward primer (Applied Biosystems, Foster City, CA) and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1.5 mmol/L MgCl₂, 1X PCR gold buffer, and 1U AmpliTaq Gold (PE Biosystems, Foster City, CA). Fluorescently FAM-labeled forward primers (Applied Biosystems) were used for LOH analysis. The cycling parameters were the following: 95°C for 7 minutes, 40 cycles of 94°C for 1 minute, specific annealing temperature for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The annealing temperature for each primer pair was 57°C except for D9S66, which was 54°C. PCR products were diluted tenfold and then separated by capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). After electrophoresis, the raw genotype files were analyzed using GeneScan 3.5 analysis software (Applied Biosystems). LOH was defined as complete or nearly complete absence of one allele (>75%), as evidenced by direct visualization. Reactions were done in triplicate. DNA samples homozygous at the marker of interest were considered noninformative for LOH determination. The smallest overlapping region (SOR) was defined as the smallest region comprising common spanning deletion in the majority of tumors. Based on the SOR findings, DPC samples were subdivided into three categories and referred to as LOH (cases with loss of heterozygosity in at least one informative locus); *HomDel* (cases with loss of both alleles); or *ROH* (cases with retention of heterozygosity in all informative loci).

Mutational Analysis of KLF4

PCR primers were designed using the Primer3 program (<http://frodo.wi.mit.edu/primer3/>) on the flanking regions of coding exons of the KLF4 gene. GeneID 9314 was used as reference sequences (primer sequences available on request) for KLF4. The annealing temperature was 57°C. The mutational status was determined by direct sequencing as follows: PCR product was purified using Montage PCR96 Cleanup Kit (Millipore, Billerica, MA). Six μ L of purified product were then sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Ap-

plied Biosystems). The sequencing reaction contained 3 μ L of purified PCR product, 2 μ L of BigDye Terminator Mix, 1.5 μ L of primer (3 pmol), and 9 μ L of BigDye Terminator v3.1 Sequencing Buffer 5X. Thermal cycling conditions were 30 cycles at 96°C for 10 seconds, at 50°C for 5 seconds, and at 60°C for 3 minutes. Unincorporated primers and dye terminators were removed using Montage-SEQ96 Sequencing Reaction Cleanup Kit (Millipore). Eight μ L of purified samples with 8 μ L of Hi-Di Formamide (Applied Biosystems) were denatured at 95°C for 3 minutes. Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Reverse Transcription PCR of the KLF4 and RGS3 Genes

Four μ L of total RNA (approx. 200 ng) were used for cDNA synthesis, using ImProm-II Reverse Transcription System (Promega, Madison, WI). A measure of 3 μ L of the resulting cDNA was included in each PCR reaction. Primer sequences for the respective cDNA were as follows: 5'-TTGGCTTCGTTTCTTCTCTCGTTGACTTT-3' and 5'-CGGAGGATGGGTCAGCGAATT-3' for KLF4; 5'-CGGGC-AAGGCAGACAA-3' and 5'-GCCTTGGATGCCATCT-3' for RGS3; 5'-GGTGAAGGTCGGAGTCAACGGA-3' and 5'-CCATGGTGGTGAAGACGCCAGTGGACTC-3' for GAPDH. Annealing temperatures were 55°C per KLF4 and GAPDH and 57°C for RGS3. PCR products were separated by electrophoresis through an ethidium bromide-stained 2% agarose gel and visualized in UV light. Gels were photographed and the bands were scanned as digital peaks. The areas of the peaks were calculated in arbitrary units using the public domain NIH Image software from the National Institutes of Health (<http://rsb.info.nih.gov/ij/>). The value of GAPDH mRNA was used as a normalizing factor to evaluate the relative expression levels of KLF4 mRNA.

Sodium Bisulfite Sequencing of KLF4 Promoter

DNA methylation was analyzed by sodium-bisulfite sequencing, using EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to manufacturer's instructions. Bisulfite deaminates and converts cytosine residues to uracil, leaving methylated cytosines unmodified.

A 184-bp fragment in the KLF4 promoter was amplified from bisulfite-modified DNA. Primers (forward: 5'-GATTATAAATTAAGGGGAGAGTGGG-3'; reverse: 5'-TCCCTAAAAATAACCATATACCAAAA-3') were designed using MethPrimer Software (<http://www.urogene.org/methprimer/index1.html>) in a region lacking CpG dinucleotides to amplify both methylated and unmethylated alleles. The amplified fragment (annealing temperature, 57°C) spanned from nucleotide-2341 to nucleotide-2157, corresponding to KLF4 transcription start site and included 10 CpG sites. PCR products were sequenced as previously described.

KLF4 Protein Expression by Immunohistochemistry

DPCs were analyzed by tissue microarrays constructed using the ATA 100 Chemicon International system. Two areas of normal tissue and three different tumor areas represented each sample in the tissue microarrays. Two 5- μ m-thick tissue microarray sections were placed onto SuperFrost Plus glass slides and immunostained in an automated system (BenchMark XT, Ventana Medical Systems, Tucson, AZ). The commercially available rabbit polyclonal anti-GKLF antibody (H-180, Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:150. Normal intestinal mucosa specimens were used as positive control for KLF4 expression; pancreatic cells were considered immunopositive when staining intensity was comparable to that of intestinal cells. The IHC results were scored as positive, when nuclear staining was present in more than 10% of cells or negative when nuclear staining was absent or present in less than 10% of tumor cells.

KLF4 Functional Study

Cell Line and Culture

The human pancreatic cancer cell line PANC-1 and BxPC3 were a generous gift of Dr. Gary Schwartz (Memorial Sloan-Kettering Cancer Center, New York, NY) and were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Plasmid Construction and Transfection

The full-length KLF4 cDNA was subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen), and the insert was verified by sequencing. For transfection, PANC-1 or BxPC3 were plated into either 12-well or 48-well plates and allowed to adhere for 12 hours. Lipofectamine 2000 (Invitrogen) was used for transfecting the cells with either pcDNA3.1-KLF4 or pcDNA3.1 alone. Cells were cultured for 72 (or 96) additional hours after transfection.

Antibody and Western Blotting

Whole-cell lysates were prepared by directly lysing cells in hot Laemmli sample buffers (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromo-phenol blue, 0.125 M Tris-HCl). For Western blot analyses, 30 μ g of cell lysate were diluted on sample buffers and resolved on 8% SDS-PAGE, transferred to a PDVF membrane and probed with anti KLF-4, anti-p21 (Santa Cruz Biotechnology), anti-cyclin D1 (Cell Signaling) or anti α -tubulin antibodies (Sigma-Aldrich, St. Louis, MO).

Proliferation Assay

Quantification of cell growth was determined in triplicates and in quadruplicates for the 72- and 96-hour experiments, respectively, using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). The absorbance at 490 nm was measured using a microplate reader.

Cell Count Assay

At the end of the transfection treatment, cells were trypsinized and counted by using a hemocytometer.

Trypan Blue Assay

At the end of the transfection treatment, cells were trypsinized, stained with trypan blue, counted using a hemocytometer, and scored as dead or alive.

TUNEL Assay

A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using an Apoptosis Detection Kit (Roche, Werk Penzberg, Germany) in accordance with the manufacturer's instructions. Slides were analyzed under a fluorescent microscope. TUNEL-positive (GFP-positive) nuclei were counted in three randomly selected microscopic fields per slide and were expressed as a percentage of the total nuclei counted. At least 1000 nuclei were counted on each slide.

Statistical Analysis

Data were summarized using descriptive statistics, including means and SDS for continuous data or proportions for categorical data. All *P* values were two-sided. Univariate methods were carried out using the one-way analysis of variance statistical test for continuous data. The differences were considered statistically significant when the *P* value was less than 0.05. KLF4 expression and frequency of LOH in KLF4 IHC and KLF4 mRNA were also compared using either the Pearson χ^2 test or Fisher's two-tailed exact test, as appropriate. The functional studies data were analyzed for significance by either the Student's *t*-test (two-tailed) or the stratified χ^2 test. The differences were considered statistically significant when *P* < 0.05.

Figure 1. A: Example of retention of heterozygosity. Samples are in heterozygosity for the specific microsatellite, and the peaks represent each allele amplification product. **Asterisks** indicate the peaks chosen for the LOH analysis. LOH analysis was conducted by measuring the ratio between the two peak fluorescence values. LOH was present if the ratio was below 0.5 or above 1.5. **B:** Example of LOH on D9S105. **C:** Pattern of LOH on 9q in DPCs. (**Black circles** are LOH cases; **grey circles** are cases with homozygous deletions; **white circles** are cases with retention; **white squares** are cases with instability; - indicates noninformative cases). The SOR region is highlighted in **grey**.

Results

A 9q Region of Frequent Allelic Loss Characterizes Both DPCs and PanINs

A panel of 40 paired normal-tumor DNA samples from DPCs and 6 PanINs were assayed for LOH using 9 polymorphic microsatellites mapped to chromosome 9q. The results are summarized in Table 2.

In at least one locus, LOH was found in 67.5% of tumor samples (27/40) and in all PanIN samples (Figure 1 and Figure 2). LOH frequencies varied among the markers from 22% (D9S1823 at 9q33.2) to 51% (D9S105 at 9q31.3) in DPCs and from 0 (D9S1823 and D9S66 at 9q33.2 and 9q34.2) to 83% (D9S105 at 9q31.3) in PanINs. In 24 cases (60%) there were interstitial allelic deletions, thus defining the SOR at 9q31.1–31.3, flanked by microsatellites D9S127 and D9S105 (Figure 1C). The same SOR was also present in all PanIN samples (Figure 2).

A homozygous deletion at SOR was detected in 5 of 37 informative cases of DPC (13.5%) and in 2 of 6 PanINs. The microsatellite D9S105 appeared to be the most sensitive marker in detecting this deletion (Figure 3A).

KLF4 Gene Expression is Associated with 9q Chromosomal Status and Significantly Decreases in DPC Compared to Normal Pancreatic Tissue

KLF4 mRNA was not detected in 71% of DPC cases (25/35). KLF4 expression was absent in 100% of cases in HomDeL (5/5) (Figure 3B); in 85.6% of cases in LOH (12/15); and in 46.6% of cases with RHO (7/15). The

overall expression pattern was significantly associated ($P = 0.002$) with the genomic asset, indicating a strong relationship between 9q31.1–3 loss and lack of KLF4 expression (Figure 3D). The LOH analysis was not conducted on the normal pancreatic ducts, under the assumption that they do not carry any chromosomal alterations.

All normal cases showed similar levels of mRNA (Figure 3C). Strong statistical significance ($P = 0.002$) was found to characterize the difference in KLF4 expression between all DPCs and normal tissue. In fact lack of KLF4 mRNA was detected in 71% of DPC samples (25/35), whereas 100% of normal pancreatic ducts (6/6) showed KLF4 expression (Table 3). No mRNA was available for the six PanIN samples.

Case N°	1	2	3	4	5	6
D9S152	○	□	○	○	○	○
D9S127	●	—	○	○	●	□
D9S53	○	●	□	—	○	○
D9S105	●	○	○	●	□	●
D9S106	○	●	○	○	—	○
D9S1823	○	○	○	○	○	○
D9S125	○	●	●	○	●	○
D9S1199	○	○	●	○	○	○
D9S66	○	○	○	○	○	○

Figure 2. Pattern of LOH on 9q in PanINs (**black circles** are cases with LOH; **grey circles** are cases with homozygous deletions; **white circles** are cases with retention; **white squares** are cases with instability; — indicates noninformative cases). The SOR region is highlighted in **grey**.

Figure 3. **A:** Schematic representation of RGS3 and KLF4 loci. **B:** Example of RT-PCR in 5 *HomDel* and 5 *LOH* cases. **C:** Expression of KLF4 in normal pancreatic ducts. **D:** mRNA expression analysis of the KLF4 gene in *HomDel*, *LOH*, *ROH*, and normal ducts. GAPDH was chosen as an internal control gene. Each histogram represents the ratio of the KLF4 and the GAPDH level of mRNA expression. There is a significant association with a P value = 0.002 between the amount of KLF4 mRNA expression and genomic status.

RGS3 Gene Expression Is not Associated with 9q Chromosomal Status

RGS3 expression was absent only in 25% of DPCs (8/33), and there is no significant difference regarding the 9q chromosomal status. In fact, RGS3 mRNA was not detected in 20% of the *HomDel* cases (1/5) (Figure 3B); in 28.6% of cases with *LOH* (4/14); or in 21.4% of *ROH* cases (3/14). Based on these data, it is unlikely that RGS3 could be involved as a tumor suppressor gene in DPC.

The KLF4 Gene in DPC

We subsequently investigated KLF4 in more detail, because its expression was absent in all *HomDel* cases and in the majority of *LOH* cases. The entire coding region of KLF4 gene was directly analyzed in 17 microdissected primary tumors: 5 *HomDel* (T-1, T-2, T-3, T-23, T-27); 9

Figure 4. **A:** Five samples of *HomDel* could not be amplified for any of the genomic coding exons of the KLF4 gene, whereas all *LOH* and *ROH* samples were successfully amplified (figure shows only a few representative samples). **B:** The Braf gene was successfully amplified in all five *HomDel* samples and in a few representative *LOH* and *ROH* samples as a control for the genomic DNA used to support the result of a KLF4 locus homozygous deletion.

LOH (T-6, T-7, T-13, T-19, T-28, T-30, T-47, T-53, T-67); and 3 *ROH* (T-24, T-33, T-75). All five *HomDel* cases fail to amplify any KLF4 coding sequences, whereas all four coding exons were successfully amplified and sequenced in both *LOH* and *ROH* cases (Figure 4, A and B). No mutations were found in these last cases. One sample (T-19) showed 1 synonymous single-nucleotide polymorphism on codon 154 (gcg > gct).

To further understand the mechanisms responsible for the loss of KLF4 expression in cases of DPC, we studied the methylation status of the KLF4 promoter region by bisulfite-sequencing PCR on DNA extracted from 12 microdissected DPC specimens (7 *LOH* KLF4 - and 5 *ROH* KLF4+). Sequencing of bisulfite is based on the selective deamination of cytosines to thymine following bisulfite treatment and the sequencing of subsequently generated PCR products. In contrast to unmethylated cytosine, the 5-methylcytosine does not react with bisulfite and can therefore be distinguished. The loss of cytosines to thymine along the sequence after bisulfite treatment therefore indicates the unmethylated status of the original sequence (Figure 5B). Only two of the seven KLF4-cases (28.6%) (T-19, T-76) show the presence of cytosine nucleotides in the CpG island (T-XX is shown in Figure 5C) and were considered methylated. None of the KLF4+ cases were methylated. These results suggest that hypermethylation may justify the loss of KLF4 expression in a minority of DPC samples.

Significant Loss of KLF4 Protein Expression in DPCs and PanINs

Immunohistochemistry was performed in 6 PanINs and in 38 DPCs, including 17 of those analyzed for RNA expression. Unlike normal intestinal epithelial cells, normal pancreatic ducts showed nuclear staining in only a few scattered cells. Three of six PanINs were negative for KLF4 immunostaining (Figure 6A), whereas the remaining three showed a nuclear positive staining (Figure 6B). The PanIN sample with the homozygous genomic deletion lacked expression of the KLF4 protein. Of 38 (86.8%) DPCs, 33 lacked KLF4 protein expression (Figure 6C), whereas 5 cases exhibited strong nuclear staining (Figure 6D).

Table 3. Difference in KLF4 Expression between 35 Ductal Pancreatic Carcinomas and 6 Normal Tissues*

	KLF4 mRNA +	KLF4 mRNA -	Total
Ductal pancreatic carcinoma	10 (29%)	25 (71%)	35
Normal pancreatic ducts	6 (100%)	0 (0%)	6
Total	16	25	41

*71% of ductal pancreatic carcinomas had no expression of KLF4 compared to 0 in normal pancreatic ducts (P value = 0.002).

Figure 5. KLF4 promoter methylation analysis. **A:** A fragment of KLF4 5' UTR showing original nucleotide sequence before bisulfite treatment; cytosines of 3 CpG dinucleotides are circled with an asterisk. **B:** Electropherogram of an unmethylated case. **C:** Electropherogram of a methylated case.

Matching of mRNA and IHC expression data in 17 cases showed a significant association between IHC and mRNA data (P value = 0.03); 14 DPC cases lacked KLF4 protein expression and 3 showed positive nuclear staining (Table 4). Of the 14 KLF4-negative samples, 11 also

Figure 6. Immunostaining with anti-KLF4 antibody: negative (A) and positive (B) PanINs; negative (C) and positive (D) DPCs (400 $\times$ magnification).

Table 4. Matching of mRNA and IHC Expression Data*

	KLF4 mRNA –	KLF4 mRNA +	Total
KLF4 IHC –	11 (79%)	3 (21%)	14
KLF4 IHC +	0 (0%)	3 (100%)	3
Total	11	6	17

*Of the samples, 17 showed matching of mRNA and IHC expression data. 14DPCs lacked KLF4 protein expression (IHC –), and 11 cases lacked KLF4 mRNA (mRNA –); 3 expressed KLF4 mRNA (mRNA +). The remaining 3DPCs expressing the KLF4 protein (IHC +) were also KLF4 mRNA + (P = 0.03).

lacked KLF4 mRNA expression, whereas in 3 cases (21%) the absence of KLF4 protein was associated with normal levels of KLF4 mRNA, thus suggesting the involvement of some post-translational events.

KLF4 Controls Proliferation and Viability in Functional Studies

To address the role of KLF4 in pancreatic cells proliferation directly, we transiently overexpressed the full-length KLF4 cDNA in PANC-1, a cell line derived from a human pancreatic carcinoma of ductal cell origin. PANC-1 is considered a good *in vitro* model for DPC and, in our hands, did not express detectable level of endogenous KLF4 (Figure 7D, first panel). To rule out the possibility that the absence of KLF4 expression in PANC-1 is due to methylation, we analyzed the methylation status of the KLF4 promoter in DNA extracted from PANC-1 and KLF4; the results showed that they were not methylated (data not shown). On PANC-1 transfection with KLF4-expressing or control vector, cell proliferation was assessed by either cell count or the MTT proliferation assay. Indeed, KLF4 over-expression induced a significant decrease of

Figure 7. Effect of KLF4 on cell proliferation and viability. The following assays were performed on PANC-1 cells 72 hours after transfection with either KLF4 or the empty-vector. **A–B:** Cell proliferation was assayed by either cell count or the MTT proliferation assay; columns represent mean of two independent experiments done in triplicate; bars, SD. **C:** Western blot analysis checked the expression level of KLF4, p21 and cyclin D1. Tubulin was used as loading control. **D:** Cell death was determined by trypan blue assay. Values are presented as dead/alive percent; columns represent the mean of two independent experiments done in triplicate; bars, SE * P < 0.05.

PANC-1 proliferation in both assays (Figure 7, A and B). It is interesting that in the MTT assay, the decrease in proliferation, evident already after 48 hours of transfection (P value <0.05), became even more pronounced at 96 hours with a 32.4% overall reduction in proliferation (P value <0.001) (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). There was a statistically significant decrease in cell count, with P value = 0.03. Moreover, over-expression of KLF4 in a different pancreatic cancer cell line, BxPC3, resulted in a significant decrease in cell proliferation confirming KLF4 involvement in the control of pancreatic cancer proliferation (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). Next, to investigate the mechanism by which KLF4 controls cell proliferation, we looked at the expression of two known KLF4 targets involved in cell cycle progression: p21 and cyclin D1. As shown in Figure 7C, KLF4 induced a dramatic increase in p21 and, at the same time, a strong down-regulation of cyclin D1.

Finally, to further investigate the mechanism by which KLF4 controls the proliferation of pancreatic cancer cells, we looked at cell death induction in PANC-1 overexpressing KLF4. We found that KLF4 caused a significant increase in cell death as measured by the trypan blue method (P value $1.2E-06$) (Figure 7D). Nevertheless, we performed a TUNEL assay to verify whether the increase in cell death was caused by apoptosis and did not find a significant difference (data not shown), suggesting that KLF4 contribution involves additional pathways for cell death.

Discussion

DPC is a deadly disease with a very poor prognosis: the overall survival at five years accounts for only 5% to 10% of all cases.¹ A progression model has been proposed for pancreatic carcinoma that is similar to that for the colonic adenoma-carcinoma sequence. Pre-invasive proliferative ductal lesions called PanINs are believed to represent the morphological precursors of invasive ductal carcinoma.^{35,36} Identification of novel molecular markers is crucial for the development of more successful and less toxic targeted treatment strategies.

Although some molecular events leading to DPC development are already known, the cause of this aggressive behavior is still largely unclear. DPCs are characterized by high genomic instability, resulting in complex karyotypes with a large number of numerical and structural chromosomal abnormalities. Allelic losses affecting the long arm of chromosome 9 are often encountered in many human malignancies, including bladder (71%),¹⁹ gastric (56.5%),²⁰ colorectal (25% to 50%),²¹ and esophageal carcinomas (67% to 86%).²² Two main regions have been described; they involve the 9q32 and 9q31 loci. LOH at 9q32 is associated mainly with esophageal and urothelial carcinomas, whereas 9q31 appears to be involved in colorectal and gastric cancers.

Here we report for the first time the high frequency of LOH at 9q in a series of 40 microdissected DPC samples. Using highly polymorphic microsatellite markers we were

able to define a commonly deleted region spanning 8 Mbp and mapped at 9q31.1–31.3. The overall frequency of LOH in this region was 67.5%. Moreover, the same analysis was carried out in a panel of six pre-invasive lesions, and we found the same pattern of deletion on 9q. In particular, all early pre-invasive lesions (PanINs) showed LOH at 9q31; 5 DPCs of 37 informative cases and 2 of 6 informative PanIN samples demonstrated a homozygous deletion at 9q31.3. Based on our results we can hypothesize that the 9q LOH event occurs earlier than the 9p, 17p, or 18q losses,³⁷ even though the number of pre-invasive samples analyzed was small. The putative tumor suppressor gene mapped at 9q31 is probably involved in early pancreatic tumorigenesis rather than in tumor progression. This hypothesis is further supported by similar findings reported in gastric cancer, where the loss of the 9q31 region has been significantly associated with the early stages of the disease.²⁰

A second candidate tumor suppressor gene, RGS3, also maps in this region and has also been suggested as a potential tumor suppressor.^{38–40} However, we were unable to detect any significant change in RGS3 expression within our series, thus excluding RGS3 as a major candidate tumor suppressor gene in our model.

On the contrary, we documented a loss of KLF4 mRNA expression in 71% of the DPCs analyzed. In particular, lack of KLF4 mRNA was documented in 100%, 85.6%, and 50% of cases showing homozygous deletion, LOH, and retention of heterozygosity respectively (Figure 3). This expression pattern was significantly associated with the genomic asset, indicating a strong relationship between 9q31 loss and KLF4 expression. Moreover, IHC analysis confirmed the loss of KLF4 protein expression in 86.8% of DPC samples. Overlapping results for IHC and RT-PCR were obtained in the majority of cases (14/17 cases). In three cases the presence of mRNA was not associated with protein expression, probably reflecting post-translational events (Table 4). KLF4 protein was also lacking in three of six pre-invasive lesions (Figure 6, A and B), supporting our hypothesis that KLF4 loss is an early event. However, in 2005 Prasad and colleagues conducted a microarray study on microdissected PanINs and found that KLF4 is up-regulated compared to normal pancreatic tissue. Nevertheless, this result was confirmed by RT-PCR and *in situ* hybridization in three of six (50%) and five of eleven (45%) of PanINs.⁴¹ In our study, no mRNA from PanINs was available for expression studies but we were able to conduct the LOH and IHC analyses that supported our theory of loss of the KLF4 locus and protein expression. The latter is probably due to some still unexplained post-translational events. As a matter of fact, loss of KLF4 protein expression was recently observed in 24.4% of colorectal cancers²⁸ and in 32.4% of primary gastric tumors.³¹ Down-regulation of KLF4 is also significantly associated with regulation of proliferation, differentiation, and tumorigenesis of gastrointestinal epithelium.³⁰ A recently generated conditional KLF4-knockout mouse model, specific for the gastric compartment, has shown that KLF4 can indeed function as a tumor suppressor gene; the mice developed gastric hyperplasia and polyps.⁴²

The mechanisms of drastically altered KLF4 expression are still unknown. Two human colorectal cancer cell lines have been described to carry point mutations in coding exons of KLF4,²⁸ but point mutations in KLF4 have not yet been reported in tumors and, consistently, we have not detected any mutation in the KLF4 coding sequences in our series of DPCs. The analysis of the KLF4 promoter methylation status evidenced that two of seven (28.6%) KLF4-negative cases were methylated. Our LOH analysis at chromosome 9q31.1–32 revealed genetic loss in the 89% of KLF4 not expressing DPCs (including the two methylated cases), indicating that both genetic and epigenetic changes may cause KLF4 gene inactivation in the majority of DPCs.

This evidence is also supported by three recent reports: (1) a high frequency of LOH on chromosome 9q31 was found in human gastric cancers²⁰; (2) the KLF4 locus was methylated and deleted in human gastric cancers³¹; (3) LOH and hypermethylation of the 5'-untranslated region have been associated with altered KLF4 expression and with functions in colorectal cancer.²⁸ However, alternative mechanisms for KLF4 transcriptional inactivation may occur in the remaining 11% of KLF4 nonexpressing DPCs that don't exhibit genetic loss or promoter methylation. In the colon cancer model KLF4 may, in fact, be down-regulated by either CDX2, APC, or β -catenin.^{43–45}

Post-transcriptional events, such as alternative splicing and alternative polyadenylation, could affect KLF4 protein levels and/or activity without having an obvious effect on total RNA levels. Although alternative splicing has not been yet described for KLF4, the relative usage of multiple KLF4 polyA sites varies in different mouse tissues and during development⁴⁶; the distal sites are predicted to generate isoforms that are more stable and/or more efficiently translated. Finally, it would be interesting to explore whether miRNAs-mediated inhibition of KLF4 plays a role in pancreatic cancer because KLF4 has very recently been described as a putative target of miR-143 and miR-145.^{47,48}

Currently, KLF4 has been added to a group of ambiguous cancer-related genes because its expression has been shown to increase in breast ductal carcinoma⁴⁹ and oral squamous carcinoma.⁵⁰ It has been shown that KLF4 switches between its tumor-suppressive and oncogenic modes as a function of the genetic context. Our results provide evidence for KLF4 tumor-suppressive functions in DPC, as indicated by the significant association between LOH at KLF4 locus, the loss of expression in most of the samples, the gene silencing due to promoter methylation, and the loss of protein expression through a hitherto unexplained mechanism. To further investigate a possible role of the KLF4 gene as a tumor-suppressor gene in pancreatic cancer, we performed a functional analysis in which the full-length KLF4 cDNA was transfected into a human pancreatic exocrine carcinoma cell line (PANC-1 and BxPC3), considered a good *in vitro* DPC model and, as in the majority of DPCs, not expressing KLF4 mRNA levels. The KLF4 promoter resulted in no methylation in PANC-1. It is interesting that overexpression of KLF4 significantly inhibits cell proliferation consis-

tently in its role as a tumor suppressor in pancreatic tumorigenesis. Enforced KLF4 overexpression induced in PANC-1 a dramatic increase in p21 and, at the same time, a strong down-regulation of cyclin D1, which are two important cell cycle regulators.⁵¹ These observations indicate that KLF4 acts as a tumor suppressor and confirms the results obtained by Hu W and colleagues⁵² in 2009 in lung cancer cell lines. Moreover, this result is also consistent with recent findings by Wei and colleagues²⁵ that KLF4 promotes p27Kip-mediated cell cycle arrest *in vitro* and attenuates tumor growth and metastasis in an orthotopic mouse model. This study was conducted on six different pancreatic cell lines, including PANC-1. Wei and colleagues²⁵ found that KLF4 is expressed at a reduced level in PANC-1 cells, whereas in our experiment the expression of KLF4 is absent. This discrepancy, which actually is not very strong, could be due to technical reasons. Moreover, they studied the suppressive effect of KLF4 on proliferation in FG and Bx3P cells but not in PANC-1. Their analysis on PANC-1 was limited to Western blot. However, the results they obtained from the KLF4 over-expression and proliferation studies on the pancreatic cell lines support our findings in PANC-1²⁵ and also in BxPC3. It is interesting also to indicate that the same authors³¹ demonstrated in 2005 how, in gastric cancer, KLF4 expression induces apoptosis. Our results show also a significant increase in cell death after KLF4 over-expression. Nevertheless, when we performed a TUNEL assay to verify whether the increased in cell death was caused by apoptosis, we did not find a significant difference, which suggests that the contribution of KLF4 to pancreatic cell lines involves additional cell-death pathways.

Conclusions

We have reported for the first time a high frequency of genomic losses at the long arm of chromosome 9 in primary DCP samples. An 8-Mbp region, bounded by microsatellites D9S127 and D9S105, is lost in 67.5% of DPC samples. This event seems to occur early because it is also documented in early PanINs, suggesting a possible role in the development of pancreatic cancer for at least one gene in this region.

KLF4 maps at 9q31.3 locus and its mRNA and protein are dramatically reduced in a large majority of DPCs (71% and 86.8%, respectively). However, we could not find any point mutations in the coding sequences of the gene, and promoter hypermethylation was observed in only a minority of cases (28.6%), so further investigations are necessary to completely understand the mechanisms responsible for KLF4 repression in DPCs.

Overall, KLF4 represents a novel gene which, being down-regulated in pancreatic cancer, could be evaluated in the development of therapeutic gene therapies for malignancies intended to be achieved through targeted gene delivery or targeted gene transcription. The use of gene substitution in cancer therapy is far from being complete; the finding of a drug that can take a suppressed gene and cause it to be re-expressed is rare.

Nevertheless, it has recently been reported⁵³ that a specific drug (RS5444) being tested in a phase 1/2 clinical trial to treat anaplastic thyroid cancer might be useful for treating other cancers. This drug induces the transcription of many genes and in thyroid cancer induces the expression of p21, which is one of the target genes of KLF4. Therefore, such findings suggest further testing of these classes of drugs, which could have significant effects on cancer biology.

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