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KLF4 α Upregulation Promotes Cell Cycle Progression and Reduces Survival Time of Patients with Pancreatic Cancer

DAOYAN WEI^{*}, LIWEI WANG[‡], MASASHI KANAI^{*}, ZHILIANG JIA^{*}, XIANGDONG LE^{*}, QIANG LI^{*}, HUAMIN WANG[§], and KEPING XIE^{*,||}

^{*}Department of Gastrointestinal Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

[§]Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

^{||}Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

[‡]Department of Medical Oncology and Shanghai Key Laboratory of Pancreatic Diseases, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, People's Republic of China

Abstract

Background & Aims—Krüppel-like factor (KLF)4 is a transcription factor associated with tumor suppression and oncogenesis. KLF4 suppresses pancreatic tumorigenesis by unknown mechanisms; we investigated alterations that might affect KLF4 function and lead to tumor formation.

Methods—We identified different isoforms of KLF4 in pancreatic cancer cells by reverse transcriptase-PCR, cloning, and DNA sequence analyses. We constructed vectors to express the isoform KLF4 α and characterize its function. Using real-time PCR, immunoprecipitation, and immunohistochemical analyses, we assessed expression of KLF4 α in pancreatic cancer cell lines and tumor tissue samples; xenograft models were used to determine the effect of KLF4 α on pancreatic tumorigenesis.

Results—We identified 4 KLF4 isoforms in human pancreatic cancer cells, designated KLF4 α , KLF4 β , KLF4 γ , and KLF4 δ . KLF4 α localized primarily to the cytoplasm; its protein and mRNA were upregulated in pancreatic cancer cell lines with high metastatic potential and human pancreatic tumors, compared with normal pancreatic tissue. Transgenic expression of KLF4 α reduced expression of p27Kip1 and p21CIP1, promoting cell cycle progression and *in vivo* tumor formation by pancreatic cancer cells. Increased expression of KLF4 α in pancreatic tumor tissue

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Correspondence: Keping Xie or Daoyan Wei, Department of Gastrointestinal Medical Oncology, Unit 426, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-2828; Fax: (713) 745-1163; kepxie@mdanderson.org or dwei@mdanderson.org.
D.W., L.W., and M.K. contributed equally to this work.

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was inversely correlated with overall time of survival in patients with stage II pancreatic ductal adenocarcinoma.

Conclusions—We identified a splice variant of KLF4 (KLF4 α) that is upregulated in aggressive pancreatic cancer cells and human pancreatic tumor tissues. Increased expression promotes growth of pancreatic tumors in mice is associated with reduced survival times of patients.

Keywords

proliferation; pancreatic cancer; cell cycle regulation; prognosis

Pancreatic cancer is currently the fourth leading cause of cancer-related deaths in the United States.¹ Although the etiology and pathogenesis of pancreatic adenocarcinoma remain unclear, heterogeneous genetic and epigenetic alterations play important roles in pancreatic cancer development and progression.^{2,3} More recently, a comprehensive pancreatic cancer genome project found that pancreatic adenocarcinoma cells harbored average 63 intragenic mutations or amplifications/homozygous deletions clustered in 12 signaling pathways.⁴ Continued identification of signature gene alterations in pancreatic cancer cells will provide a conceptual framework to guide future analyses of this complex disease and the development of strategies for early detection and effective treatment of it.

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor. KLF4 mRNA expression is found primarily in postmitotic, terminally differentiated epithelial cells in organs such as the skin and gastrointestinal tract.^{5–6} In cell culture, KLF4 expression can be increased by serum deprivation, contact inhibition, and DNA damage,^{7–8} and KLF4 is required for the maintenance of genetic stability.⁹ Recently, reduced expression of KLF4 has been reported in various tumors, and restoration of KLF4 expression can induce growth arrest in colon cancer cells and apoptosis in bladder and gastric cancer cells and leukemia cells.^{10–14} Furthermore, accumulating clinical evidence suggests that KLF4 functions as a tumor suppressor, and studies have found genetic and epigenetic alterations of the KLF4 gene in gastrointestinal cancers.^{11,15} Conversely, KLF4 expression is increased in primary breast ductal carcinoma and oral and skin squamous carcinoma cells,^{16,17} suggesting that KLF4 is important to the development and progression of these tumors.^{18,19} In a previous study, we found that KLF4 has a tumor-suppressive function in pancreatic cancer cases and that induction of p27^{Kip1} expression contributes to this function.²⁰ However, whether genetic and epigenetic alterations of KLF4 occur in patients with pancreatic cancer and, if so, the underlying molecular mechanisms of these alterations remain unknown.

In the present study, we identified four KLF4 splicing variants in human pancreatic cancer cells. We found that the KLF4 α isoform protein in particular was located primarily in the cytoplasm of pancreatic cancer cells, and additional results indicated that KLF4 α has an oncogenic function and that altered KLF4 α expression may contribute to the development and progression of pancreatic cancer.

Materials and Methods

Detailed materials and methods are described in the Supplementary Methods.

RNA Extraction, Reverse Transcriptase-Polymerase Chain Reaction, and Northern Blot Analysis

Total RNA or mRNA was extracted from cell culture or tumor tissues, reversely transcribed into cDNA for PCR analysis or directly used for Northern blot analysis as described previously²⁰ and in the Supplementary Materials and Methods.

Construction of KLF4 α , KLF4 Expression Vectors and stable cell line generation

Standard recombinant DNA technique was used to construct related vectors, and some of resultant vectors were used to generate stable cell lines as described in the Supplementary Methods.

Quantitative Real-Time PCR and TissueScan Oncology Panel

Total RNA was reversely transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The cDNA products were used in qPCR analyses of gene expression using PCR primer and probe sets custom-designed or purchased from Applied Biosystems (Supplementary Materials and Methods) and relative RNA-expression calculations were performed using a commercially available software programs (SDS version 1.2; Applied Biosystems) as described in Supplementary Materials and Methods.

Human KLF4 α Antibody Generation and Characterization

A specific antiserum against human KLF4 α was produced (GN330), and described in the Supplementary Materials and Methods.

Immunocytochemistry

For subcellular localization study, HEK293, PANC-1 or HCT-116 cells were transfected with related expression vectors, and followed by similar treatment and image acquiring procedures described previously²⁰ and in the Supplementary Materials and Methods.

Western Blot and Immunoprecipitation Analysis

To evaluate the levels of KLF4 α , KLF4, p27^{KIP1}, and p21^{CIP1} expression, cell or tissue lysates or immunoprecipitates were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunodetection was performed as described previously²²⁻²³ and in the Supplementary Methods.

Tissue Microarray Analysis

The tissue microarray construction and tissue sample information were described previously.²³ Standard immunohistochemical procedures were performed on human pancreatic tissue microarray samples, and the staining results were scored as described previously.²⁴ An overall score at or below 3 was defined as negative, whereas an overall score greater than 3 was defined as positive (see Supplementary Materials and Methods).

Cell Proliferation Assay

MTT analysis was used for evaluation of cell proliferation as described in the Supplementary Methods.

Small Interfering RNA

Human KLF4 α small interfering RNA oligos were synthetically ordered from Ambion (sense, 5'-uaacagcucaugccaccgtt-3'; and antisense, 5'-cggguggcaugagcuguuatt-3') and used for knocking down KLF4 α expression in PANC-1 or L3.3 cells following the procedures described previously.²⁰ In some experiments, siRNA was cotransfected with KLF4 or KLF4 α expression vector. Cell or protein samples were harvested at 48 h after transfection and processed for flow cytometric or Western blot analysis.

Flow Cytometric Cell Cycle Analysis

FG, BxPC-3, L3.3 or Panc02 cells were prepared for fluorescence-activated cell sorting (FACS) analysis of cell cycle distribution as described previously²⁰ and in the Supplementary Materials and Methods.

Analysis of p27^{Kip1} Promoter Activity

The proximal human p27^{KIP1} promoter p27-N-MB435 was constructed previously and used in cotransfection with pcDNA3.1, pcDNA3.1-Flag-KLF4 α and/or pcDNA3.1-Flag-KLF4 for promoter activity assay using the similar procedures described previously.²⁰

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using ChIP Assay kit (Millipore, Billerica, MA) by following the procedures described previously²⁰ and in the Supplementary Methods.

Animal models of tumor growth

Tumor cells in exponential growth phase were prepared and then injected into the subcutis or pancreas of nude mice (10 mice per group) or C57BL/6 mice (5 mice per group) (1×10^6 cells per mouse for human cells and 5×10^5 cells per site for mouse cells). The animals were killed 25 (mouse tumor) or 45 d (human tumor) after the tumor cell injection or when they seemed moribund. Primary tumor tissues were harvested, weighed and processed for further analyses.

Statistical analysis—Each experiment was performed independently at least twice with similar results; findings from one representative experiment are presented. The similar methods were used for statistical analysis of *in vitro* and *in vivo* data described previously.^{11, 20} In all of the tests, *P* values less than 0.05 were considered statistically significant.

Results

Identification of KLF4 Isoforms in human pancreatic cancer cells

In our analysis of KLF4 mRNA expression in pancreatic cancer cells using Northern blot approach, we detected several bands when using full-length human KLF4 cDNA as a probe (Figure 1A). Researchers previously observed a similar phenomenon using murine mRNA samples.⁶ To identify putative KLF4 isoforms, we first used total RNA as a template for RT-PCR analysis of KLF4 isoforms using the forward and reverse primers that cover both 5'- and 3'-UTR region of KLF4 transcript (Figure 1D). As seen in Figure 1B, several bands were observed in the pancreatic cancer cell lines tested. We then used mRNAs to amplify KLF4 again, which produced quite sharp bands in agarose gel electrophoresis (Figure 1C). We isolated, cloned, and sequenced the clones derived from the individual bands in RT-PCR product of Panc-1 cells. We confirmed that the primary and largest band was wt KLF4 (Figure 1C, wt); the band α was derived from exon 3 deletion, the band β was derived from exon 3 and 4 deletions, and the band γ was derived from partial exon 2 and 5 deletions in addition to exon 3 and 4 deletions; while the band δ was derived from exon 2–4 deletions. We designated them as the isoforms of KLF4 α , KLF4 β , KLF4 γ and KLF4 δ respectively (Figure 1C). These four isoforms are *cis*-splicing products and are summarized in Figure 1D. According to their cDNA sequences, KLF4 α , KLF4 β , and KLF4 γ are expected to encode truncated proteins with frame shift (Supplementary Figure 1A). Since KLF4 α is the largest isoform among the four identified KLF4 splicing variants, we did additional analysis and found that deduced KLF4 α polypeptide has the same 33 amino acid residuals as wild type

KLF4 at the N-terminal, but the rest amino acid sequence of KLF4 α is different from that of KLF4, including lack of NLS and DNA binding domain.¹⁹ We then decided to characterize KLF4 α by asking whether KLF4 α commonly exists in human pancreatic cancer cells. Our results showed that the transcript of KLF4 α not only existed in pancreatic cancer cells but also in other types of cancers including gastric and colorectal cancer cells (Supplementary Figure 1B & 1C).

Subcellular Localization of KLF4 α in Pancreatic Cancer Cells

To determine the subcellular location of KLF4 α , HEK293 or PANC-1 cells were transfected with a red fluorescent protein (DsRed)-tagged KLF4 α expression vector. As predicted according to its amino acid sequence lacking a nuclear localization signal, DsRed-tagged KLF4 α protein was mainly distributed in the cytoplasm, whereas GFP-tagged KLF4 protein was located in the nuclei of transfected cells (Figure 2A & 2B). In another set of experiments, we used the FLAG-KLF4 α and FLAG-KLF4 expression vectors to transfect HCT-116 cells and performed immunocytochemical analysis of FLAG-tagged proteins in the transfected cells, and similar results were obtained and shown in Supplementary Figure 2A, which were also consistent with the results of Western blot analyses of both exogenous and endogenous KLF4 α proteins using cellular cytosol and nuclear extracts, respectively (Figure 2C and Supplementary Figure 2B).

Differential Expression of KLF4 α in Pancreatic Cancer Cells and Tumor Tissue

We initially selected or designed real-time PCR primer and probe sets for the specific detection of KLF4 α and wt KLF4 at RNA level (Figure 3A), which were later used to detect KLF4 α and KLF4 in a panel of pancreatic cancer cell lines. As shown in Figure 3B, pancreatic cancer cells differentially expressed KLF4 α and KLF4. Next, we compared the levels of expression of KLF4 α in pancreatic cancer cell lines with well-characterized metastatic potential using the level of KLF4 α expression in the parental Colo357 cells as a reference. Figure 3C indicates that the cell lines with high metastatic potential tended to have increased KLF4 α expression and thereby increased the KLF4 α :wt KLF4 ratio. Interestingly, we did not find a significant correlation of KLF4 α expression with wt KLF4 expression at the RNA level among the 13 human pancreatic cancer cell lines tested in a linear regression analysis ($F = 3.54$; $P > .05$), indicating that molecular mechanisms other than the level of KLF4-wt expression determine the level of KLF4 α expression in these cancer cells. Secondly, TissueScan Oncology qPCR Arrays (OriGene Technologies), which contain a panel of normalized cDNAs prepared from pathologist-verified pancreatic tumor and normal pancreatic tissues, were used to detect the expression of KLF4 α in human pancreatic ductal adenocarcinoma tissues. We found that tumor tissue samples had elevated KLF4 α expression when using the average level of KLF4 α expression in two normal pancreatic tissue samples as a reference (Figure 3D). These results indicated that altered KLF4 α expression may be involved in the development and progression of pancreatic cancer. Thirdly, to examine KLF4 α protein expression, we generated a polyclonal antibody against KLF4 α (GN330). As shown in Figure 3E, this antibody can specifically recognize KLF4 α , but not KLF4 protein. We also performed immunoprecipitation analysis to detect endogenous KLF4 α protein expression in pancreatic cancer cell lines. We found that the cell lines tested differentially expressed KLF4 α protein (Figure 3F), and that, consistent with the results of real-time PCR analysis, the cell lines that had relatively high levels of KLF4 α mRNA expression had high levels of KLF4 α protein expression, indicating that the methods we established for the detection of KLF4 α mRNA and protein expression are reliable and closely correlated.

Direct Correlation of Increased Expression of KLF4 α with Poor Prognosis for Pancreatic Cancer

Furthermore, we examined the expression of KLF4 α in human pancreatic tissue samples using immunohistochemical analysis. We found that only 1 of the 66 normal tissue samples (2%) exhibited KLF4 α --positive staining. In contrast, we found that 15 of the 65 pancreatic tumor tissue samples (23%) exhibited KLF4 α --positive staining (Figures 4A & 4B1) ($P = .000$). We also found that of 22 patients with stage II pancreatic ductal adenocarcinoma, 4 patients had KLF4 α --positive staining; these patients had a median survival duration of only 13.4 months, whereas the 18 patients with KLF4 α --negative staining had a median survival duration of 33.4 months. Furthermore, KLF4 α --positive staining was inversely correlated with the overall survival duration in Kaplan-Meier survival analysis (Figure 4B2) ($P < .05$). Next, we examined the expression of KLF4 α and KLF4 in parallel by IHC using consecutive sections of human pancreatic cancer tissue microarray, and observed significant difference in terms of expressing patterns between KLF4 α and KLF4 in those tissues. The human pancreatic cancer tissues in general exhibit negative or weak KLF4 staining, but moderate or strong positive KLF4 α staining (Figure 4C1, $P < .001$). The representative cases are presented in Figure 4C2. Interestingly, higher percentage of KLF4 α positive staining was found in the tissues from the US Biomax's than that from MD Anderson Cancer Center. The difference of KLF4 α positivity in these two cohorts may be due the fact that US Biomax's cohort consists of cases with more advanced pancreatic cancer than that from MD Anderson Cancer cohort (Supplementary Table 1). These results suggest that altered KLF4 α expression may contribute to the development and progression of pancreatic cancer and that KLF4 α may be a useful prognostic marker for pancreatic cancer.

Effects of Modulating KLF4 α Expression on Pancreatic Cancer Cell Growth In Vitro and In Vivo

For functional analysis, we stably transduced FG and BxPC-3 cells with KLF4 α mediated by a lentiviral vector. As confirmed by real-time PCR and Western blot analyses (Figure 6C & Supplementary Figure 4A), overexpression of KLF4 α significantly promoted the proliferation of both FG and BxPC-3 cells *in vitro* by MTT assay (Figure 5A), which was correlated with accelerated cell cycle progression by FACS analysis (Figures 5B & 5C; $P < .01$: B2 vs B1 in S and G1 phases; $P < .05$: C2 vs C1 in S and G1 phases. Data not shown); and similar results were also observed in murine Panc02 pancreatic adenocarcinoma cells (Supplementary Figure 4C). On the contrary, specific knockdown of KLF4 α expression (Supplementary Figure 4B) resulted in attenuated cell cycle progression in L3.3 cells (Figure 5D, $P < .05$: D3 vs D2 in S and G1 phases. Data not shown), which have relative high level endogenous KLF4 α expression (Figure 3C & 3F). Furthermore, we found that forced expression of KLF4 α significantly promoted pancreatic tumor growth *in vivo* (Figure 5E and Supplementary Figure 4D). These results suggest that KLF4 α has an oncogenic function in pancreatic cancer cells.

Effect of KLF4 α on the Expression of Genes Key to Cell Cycle Regulation in Pancreatic Cancer Cells

Mechanistically, we initially examined the effect of KLF4 α on the promoter activity of p27^{Kip1}, which is an important negative cell cycle regulator. As shown in Figure 6A, transduction of HEK293 cells with KLF4 α significantly reduced the p27^{Kip1} promoter activity, which was consistent with reduced expression of p27^{Kip1} at mRNA level in FG cells stably transduced with KLF4 α gene (Figure 6B). Consistently, we found that forced expression of KLF4 α was associated with reduced p27^{Kip1} protein expression in both FG and BxPC3 cells *in vitro* (Figure 6C), and p27^{Kip1} mRNA and protein expressions in xenograft tumor tissues derived from FG cells (Figure 6D). This observation was further confirmed by IHC analysis of the xenograft tumor tissues, showing that increased KLF4 α

staining was associated with decreased p27^{Kip1} but increased PCNA staining (Figure 6E). Consistently, forced KLF4 α expression was also associated with reduced expression of p21^{CIP1} mRNA and protein in pancreatic cancer cells (Supplementary Figure 5A). These results indicate that KLF4 α has the opposite function of KLF4, which positively regulates p27^{Kip1} and p21^{CIP1} expression.^{20, 25, 26}

To further understand the mechanisms underlying the regulation of p27^{Kip1} expression by KLF4 α in pancreatic cancer cells, we performed p27^{Kip1} promoter activity assay by cotransfection of p27-N-MB435 reporter with KLF4 α and/or KLF4 expression vector into PANC-1 cells. We found that KLF4 induced, whereas KLF4 α reduced the promoter activity of p27-NMB435, within which three KLF4 binding sites were essential for the induction of p27^{Kip1} expression by KLF4;²⁰ When KLF4 α and KLF4 expression vectors were co-transfected, the increased p27^{Kip1} promoter activity induced by KLF4 was significantly attenuated (Supplementary Figure 5B1), and the changes of p27^{Kip1} promoter activity were consistent with the changes of p27^{Kip1} protein expression as determined by Western blotting (Supplementary Figure 5B2 & 5B3). Next, we performed reciprocal immunoprecipitation analysis, and found that KLF4 α protein interacted with KLF4 protein in FG cells (Supplementary Figure 5C1 & 5C2). Consistently, forced expression of KLF4 α in PANC-1 cells significantly reduced the DNA binding of endogenous KLF4 protein to the proximal promoter region of p27^{Kip1} gene (Supplementary Figure 5D1 & 5D2) in ChIP analysis. Finally, cotransfection of DsRed-KLF4 α with GFP-KLF4 resulted in significant cytoplasmic distribution of KLF4 protein (Supplementary Figure 6 A2 vs C1), suggesting that KLF4 α affects the sub-cellular localization of KLF4 protein.

Discussion

In the present study, we first identified four KLF4 splicing variants--KLF4 α , KLF4 β , KLF4 γ , and KLF4 δ in human pancreatic cancer cells. Second, further characterization revealed that KLF4 α protein was primarily distributed in the cytoplasm and that KLF4 α was differentially expressed in the pancreatic cancer cell lines. Third, forced expression of KLF4 α significantly promoted the proliferation and cell cycle progression of pancreatic cancer cells in vitro and the tumorigenicity in animal models. Fourth, human pancreatic tumor tissue samples exhibited elevated levels of KLF4 α protein expression, which was inversely correlated with survival duration. Finally, mechanistic studies showed that forced KLF4 α expression correlated with a significant reduction in p27^{Kip1} promoter activity and mRNA expression and reduced p27^{Kip1} and p21^{CIP1} protein expression in pancreatic cancer cells. Collectively, our results provided the first, novel insight into the alteration of KLF4 α expression in pancreatic cancer cells.

KLF4 has been associated with both tumor promotion and tumor suppression.¹⁹ In the pancreas, KLF4 plays an important role in regulation of the differentiation of pancreatic ductal epithelial cells.²⁷⁻²⁸ Recently, we found that ectopic expression of KLF4 in pancreatic cancer cells induced p27^{Kip1} expression, resulting in cell cycle arrest and cell-growth inhibition in vitro and in vivo, suggesting that KLF4 has a tumor-suppressive function.²⁰ However, a previous microarray analysis indicated that KLF4 expression was upregulated in pancreatic intraepithelial neoplasia lesions.²⁹ Although researchers have argued that upregulation of KLF4 expression in early premalignant pancreatic intraepithelial neoplasia lesions may be a defense mechanism against oncogene activation,² whether other molecular mechanisms are responsible for this contradictory phenomenon of KLF4 expression and function in pancreatic cancer remains unknown. Nevertheless, we identified four KLF4 splicing variants in human pancreatic cancer cells, and similarly, we also identified a KLF4 splicing variant in mouse pancreatic cancer cells in the present study, which shares high DNA sequence similarity with human KLF4 α (Supplementary Figure

1D). Furthermore, we found that expression of KLF4 α was increased in a subset of human pancreatic cancer cell lines and tumor tissues and that KLF4 α is primarily distributed in the cytoplasm and that interacting with KLF4 and interfering with the function of KLF4 and thus resulted in the downregulation of p27^{Kip1} and p21^{CIP1} expression might be at least in part responsible for the oncogenic function of KLF4 α in pancreatic cancer cells. These results, for the first time, provided novel insight into alteration of KLF4 expression in human pancreatic cancer cells, which may at least explain in part the discrepancy on KLF4 expression and function in pancreatic cancer cells.

Alternative splicing is a process by which the exons of the RNA produced by transcription of a gene are reconnected in multiple ways during RNA splicing. Disregulated splicing and alternative splicing can cause diseases directly or contribute to the susceptibility or severity of diseases including cancer.^{30–33} Cancer-specific splicing variants may be useful biomarkers for diagnosis or therapy.^{30–35} Authors have reported the existence of splicing variants in many tumor suppressor genes, including KLF6, p73, PTEN and P53. Significantly, Narla reported that increased alternative splicing of the KLF6 gene was associated with an increased risk of prostate cancer and that targeted inhibition of KLF6-v1 suppressed prostate cancer cell growth.^{32,33} Similarly, we found that increased KLF4 α expression was correlated with poor prognosis for pancreatic cancer, suggesting that a targeted intervention designed to alter KLF4 α expression may be a promising approach to pancreatic cancer treatment. Mechanistically, intronic and extronic splicing site mutations and altered expression of splicing *trans*-acting factors such as SR proteins and heterogeneous nuclear ribonucleoproteins reportedly are the main causes of aberrant expression of splicing variants in cancer cells.^{31,34} Additionally, a DNA polymorphism at the splice-regulatory protein-binding site is linked with aberrant expression of the KLF6 splicing variant and associated with increased prostate cancer risk.³² Recently, investigators identified KLF4 gene mutations in colorectal and gastric cancers.^{11,15} In our preliminary study, we identified a DNA polymorphism at a potential splice-regulatory protein-binding site located at intron 3 of KLF4 gene, and whether this DNA polymorphism is linked to the altered KLF4 α expression in pancreatic cancer is currently under investigation in our laboratory. In addition, pancreatic cancer is characterized by high incidence of K-ras mutation. A recent study found that oncogenic Ras signaling activation was associated with increased KLF6 alternative splicing and increased expression of KLF6-v1 in human hepatocellular carcinoma,³⁵ and thus, whether K-ras mutagenic activation is responsible for the altered KLF4 α expression in pancreatic cancer cells warrants further investigation, and the result may help explain the differential KLF4 α expression in normal and tumor tissue in the present study.

Functionally, we previously found that transduction of pancreatic cancer cells with KLF4 caused significant cell-growth inhibition owing to blockage of cell cycle progression, which was associated with upregulation of p27^{Kip1} and, to a lesser extent, p21^{CIP1} expression but downregulation of cyclin D1 expression.²⁰ In the present study, our results showed that transduction of pancreatic cancer cells with KLF4 α promoted cell cycle progression in vitro and tumor growth in vivo. Consistently, transduction of these cells with KLF4 α reduced the expression of p27^{Kip1} and p21^{CIP1}. These results indicated that KLF4 α and KLF4 have opposite functions, but the underlying molecular mechanism of KLF4 α function in pancreatic cancer remains unknown. Given that KLF4 α protein does not have a nuclear localization signal, is primarily located in the cytoplasm (Figure 2), and partially retains the N-terminal transcription activation domain of KLF4 (exon 1 and exon 2 coding region), whereas KLF4 protein is primarily located and functions in the nuclear, it is reasonable to speculate that KLF4 α may interfere with the function of wt KLF4 via direct or indirect interaction with KLF4 protein and negatively regulate p27^{Kip1} and p21^{CIP1} expression. This notion is supported by our data in Supplementary Figure 5 & 6, showing that KLF4 α

interacted with KLF4 protein and forced expression of KLF4 α resulted in distribution of KLF4 protein in the cytoplasm, and decreased its DNA binding to the p27^{Kip1} promoter region, and decreased the expression of p27^{Kip1} protein. However, the detailed molecular mechanisms that KLF4 α interferes with the function of KLF4, and whether there are other molecular mechanisms underlying the oncogenic function of KLF4 α in pancreatic cancer cells remain to be defined.

In summary, this is the first study to demonstrate the presence and altered expression of an oncogenic KLF4 splicing variant, KLF4 α , in pancreatic cancer cells, which provides novel insight into altered expression of KLF4 in pancreatic cancer. The results of our study suggest that KLF4 α might be a potential prognostic marker or therapeutic target for pancreatic cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Involvement with the manuscript:

Study concept and design: KEPING XIE, DAOYAN WEI, MASASHI KANAI

Acquisition of data: DAOYAN WEI, MASASHI KANAI, KEPING XIE

Analysis and interpretation of data: KEPING XIE, DAOYAN WEI, LIWEI WANG

Drafting of the manuscript: DAOYAN WEI, KEPING XIE

Critical revision of the manuscript for important intellectual content: KEPING XIE

Statistical analysis: LIWEI WANG, DAOYAN WEI, KEPING XIE

Obtained funding: KEPING XIE, DAOYAN WEI

Technical support: XIANGDONG LE, ZHILIANG JIA, QIANG LI

Material support: KEPING XIE, DAOYAN WEI, HUAMIN WANG,

Study supervision: KEPING XIE, DAOYAN WEI

Abbreviations used in this paper

KLF4	Krüppel-like factor 4
KLF4α	Krüppel-like factor 4 splicing isoform α
RT-PCR	reverse transcriptase-polymerase chain reaction
DMEM	Dulbecco's Modified Eagle's Medium
FCS	fetal calf serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DAPI	4'6-diamidino-2-phenylindole
GFP	green fluorescent protein

DsRed	Discosoma sp. red fluorescent protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
PCNA	proliferating cell nuclear antigen
CHIP	chromatin immunoprecipitation
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IHC	immunohistochemistry

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Figure 1.

Identification of KLF4 isoforms in pancreatic cancer cells. (A) A representative result of Northern blotting using mRNA from PANC-1 cells and a radiolabeled full-length KLF4 cDNA as a probe. Three bands were detected (arrows). (B) Total RNAs from different pancreatic cancer cell lines were used for RT-PCR analysis of KLF4 isoforms using the forward and reverse primers indicated in D. Several bands were detected in all cell lines tested. (C) mRNAs extracted from the cells indicated were used for RT-PCR analysis again; the individual sharp bands derived from PANC-1 cells in agarose gel were isolated and cloned into the pGEM-T easy vector. Plasmid DNAs extracted from at least six clones derived from each RT-PCR DNA band were DNA-sequenced in both directions, with the results depicted in D. (D) Four KLF4 isoforms caused by *cis*-alternative splicing of KLF4 mRNA--KLF4α, KLF4β, KLF4δ, and KLF4γ--were identified in pancreatic cancer cells, the corresponding splicing site sequences are listed under each diagram, the letters in uppercase represent the exon part, while the letters in lowercase represent the intron part. The number of amino acid residuals deduced from the open reading frame of the individual isoform cDNAs is shown under the name of each isoform on the left side.

Figure 2.

Subcellular localization of KLF4 α protein in culture cells. (A) An DsRed-tagged KLF4 α expression vector was transfected into HEK293 cells and photographed under a fluorescent microscope for DsRed imaging (A1) and DAPI nuclear staining (A2) 48 h after transfection. An EGFP-tagged KLF4 expression vector was transfected into HEK293 cells as a control (A4). The A3 is the merge of A1 and A2, while A6 is the merge of A4 and A5. (B) Similarly, both DsRed-tagged KLF4 α and EGFP-tagged KLF4 expression vectors were transfected into PANC-1 cells, respectively, and the corresponding images are shown in B1–B6. (C) Both nuclear and cytoplasmic proteins were extracted from HCT-116 cells 48 h after transfection with FLAG-tagged KLF4 α or FLAG-tagged KLF4 expression vector. The protein samples were used for Western blotting of FLAG-tagged protein expression. Protein sample loading was monitored by detection of GAPDH and Histone H1 level, respectively.

Figure 3.

KLF4α expression in pancreatic cancer cells. (A) Real-time PCR primer and probe sets targeting the boundary of exons 2–4 or boundary 2–3 of KLF4 mRNA were selected or designed and shown to specifically detect KLF4α or wt KLF4 mRNA in FG cells transduced with control, KLF4 or KLF4 expression vector as indicated on the X-axis. (B) Differential expression of KLF4α and KLF4 in pancreatic cancer cell lines as determined using a real-time PCR under the conditions shown in A. (C) Real-time PCR analysis of relative KLF4α and KLF4 expression in pancreatic cancer cell lines with different metastatic potential. Note: the expression levels in the parental Colo357 cell line were used as a reference for comparison. (D) Real-time PCR analysis of KLF4α expression in human pancreatic tissue samples using a TissueScan Oncology qPCR Array of a panel of prenormalized cDNA samples. The relative KLF4α expression in pancreatic tumor (T) tissue was normalized according to the mean level of KLF4α (used as a reference) in two normal pancreatic tissue samples (N3 and N4). (E) Western blot analysis of specificity of anti-KLF4α antibody (GN330). Protein samples from HEK293 cells transfected with KLF4, KLF4α or Flag-tagged KLF4α expression vector were used in immunoblot analysis with an H-180 (anti-KLF4) or KLF4α antibody. The results indicated that GN330 antibody specifically recognized KLF4α protein (2, lanes 1 and 4) but not wt KLF4 (2, lane 3), whereas the H-180 antibody recognized both KLF4 and KLF4α protein (1, KLF4α: lanes 1 and 4; KLF4: lane 3). (F) Immunoprecipitation analysis of endogenous KLF4α protein expression in pancreatic cancer cells. Equal amounts of total cell lysates were immunoprecipitated with the H-180 antibody, and the precipitates were used for Western blot analysis with the KLF4α primary (GN330) antibody. Protein samples from HEK293 cells transfected with a KLF4α expression vector were used as positive controls (Po-Ctr) in Western blot analysis.

Figure 4.

Expression of KLF4 α protein in pancreatic tissue and its association with survival. (A) Immunohistochemical analysis of KLF4 α protein expression in pancreatic tissue samples with representative results showing KLF4 α -negative (1, normal pancreatic tissue and 2, pancreatic tumor tissue) and -positive (3, pancreatic tumor tissue) staining. (B) Most of the normal samples (Non-T) exhibited no detectable KLF4 α staining, whereas 23% of the pancreatic tumor samples (Tumor) exhibited KLF4 α -positive staining. Pearson's two-tailed χ^2 test showed a significant statistical difference in KLF4 α protein staining between the two tissue types (B1, $*P < .001$). KLF4 α -positive staining was associated with reduced durations in a Kaplan-Meier survival analysis of 22 patients with stage II pancreatic ductal adenocarcinoma (B2, $P < .05$; log-rank test). (C). IHC analysis of KLF4 α and KLF4 protein expression in consecutive tissue microarray sections using KLF4 α (GN330) and KLF4 antibodies, which specifically recognized KLF4 α and KLF4 protein, respectively (see Supplementary Figure 3). The difference between KLF4 α and KLF4 protein expression was significant (C1, $*P < .001$ by Pearson's two-tailed χ^2 test), and the representative staining results were shown (C2, Case#1: Negative KLF4 staining, strong positive KLF4 α staining; Case #2: Weak KLF4 staining, positive KLF4 α staining).

Figure 5.

The effects of modulating KLF4α expression on cell growth and cell cycle progression in pancreatic cancer cells. (A) MTT analysis of FG (1) and BxPC-3 (2) cell proliferation in the culture of complete DMEM containing 2% FCS for 48 h. (B and C) Cell cycle analysis of FG (B) and BxPC-3 (C) cells after culture in complete DMEM containing 2% FCS for 24 h. (D) Cell cycle analysis of L3.3 cells at 48 h after mock, control siRNA or KLF4α siRNA transfection. (E) *In vivo* pancreatic tumor growth. FG cells with forced KLF4α expression grew larger tumors than did FG cells transduced with a GFP control gene in orthotopic mouse models (1, representative tumor sizes; 2 & 3, mean tumor weight of two independent experiments). **P* < .05 versus GFP control.

Figure 6. KLF4 α regulates cell cycle-related gene expression. (A) p27^{Kip1} promoter activity in HEK293 cells was measured 48 h after transduction of the cells with KLF4 α or a control vector using a dual luciferase assay. (B) Real-time PCR analysis of p27^{Kip1} expression in FG cells stably transduced with KLF4 α or a GFP control gene and cultured in complete DMEM containing 2% FCS for 24 h. (C) Overexpression of KLF4 α correlated with reduced expression of p27^{Kip1} protein in FG and BxPC-3 cells stably transduced with the KLF4 α gene and cultured in complete DMEM containing 2% FCS for 24 h in Western blot analysis. (D) Forced expression of KLF4 α correlated with reduced expressions of p27^{Kip1} mRNA (1) and protein (2) in the pancreatic tumor tissue samples derived from the orthotopic mouse model. (E) Representative results of IHC staining for the detection of KLF4 α , p27^{Kip1} and PCNA expressions in the pancreatic tumor tissue samples derived from FG xenograft tumors, and lower panel of graphs show the average scores of IHC staining evaluated from 6 independent high-power fields (HPF, $\times 400$) for each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control or GFP.