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TUMOR-SPECIFIC EXPRESSION AND ALTERNATIVE SPLICING OF THE COL6A3 GENE IN PANCREATIC CANCER

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Abstract

Introduction—Pancreatic ductal adenocarcinoma (PDA) is a highly lethal disease in which a prominent desmoplastic reaction is a defining characteristic. Fibrillar collagens, such as collagen I and to a lesser extent, collagen III and V comprise the majority of this stromal fibrosis. Type VI collagen (COL6) forms a microfibrillar network associated with type I collagen fibrils. The expression of COL6 has been linked to inflammation and survival. Importantly, tumor-specific alternative splicing in COL6A3 has been identified in several cancers by genome exon arrays. We evaluated the expression and localization of COL6A3 in PDA and premalignant lesions and explored the presence of alternative splicing events.

Methods—We analyzed paired PDA-normal (n=18), IPMN (n=5), pancreatic cystadenoma (n=5), and eight PDA cell lines with RT-PCR, using unique primers that identify total COL6A3 gene and alternative splicing sites in several of its exons. Western blot analysis and immunohistochemistry were used to analyze the expression levels and localization of COL6A3 protein in the different lesions, and in two animal models of PDA.

Results—COL6A3 protein levels were significantly upregulated in 77% of the paired PDA-adjacent tissue examined. COL6A3 was mainly present in the desmoplastic stroma of PDA, with high deposition around the malignant ducts and in between the sites of stromal fatty infiltration. Analysis of the COL6A3 splice variants showed tumor-specific consistent inclusion of exons 3 and 6 in 17 of the 18 (94%) paired PDA-adjacent tissues. Inclusion of exon 4 was exclusively tumor-specific, with barely detectable expression in the adjacent tissues. IPMN and pancreatic cystadenomas showed no expression of any of the examined exons. Total COL6A3 mRNA and exon 6 were identified in six PDA cell lines, but only two cell lines (MIA PACA-2 and ASPC-1) expressed exons 3 and 4. In both the xenograft and transgenic models of PDA, COL6A3 immunoreactivity was present in the stroma and some PDA cells.

Conclusions—We describe, for the first time, a dynamic process of tumor-specific alternative splicing in several exons of stromal COL6A3. Alternatively spliced proteins may contribute to the

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etiology or progression of cancer and may serve as markers for cancer diagnosis. Identification of COL6A3 isoforms as PDA-specific provides the basis for future studies to explore the oncogenic and diagnostic potential of these alternative splicing events.

Keywords

pancreatic cancer; COL6A3; stroma; microenvironment

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of adult cancer deaths in the United States (1). In 2010, it is estimated that there will be 43,140 new cases of pancreatic cancer in the United States and 36,800 deaths from the disease (1). PDA is often diagnosed at an advanced stage because it is an aggressive disease and, sadly, there are no definitive methods for early detection. The overall 5-year survival rate is less than 5% and more than 85% of the tumors have infiltrated into adjacent organs or have metastasized at the time of diagnosis (2,3). Therefore, there is an urgent need to improve our understanding of the basic molecular mechanisms that contribute to the aggressiveness of PDA and to develop more effective therapies.

Recently, the tumor microenvironment has gained much attention as a critical determinant of tumor progression and clinical outcome. Several cell types compose the tumor microenvironment including fibroblasts, immune cells, endothelial cells and adipocytes. Cancer associated fibroblasts are myofibroblast-like cells that induce the formation of a desmoplastic reactive stroma which promotes tumor growth (4).

The majority of stromal fibrosis is composed of fibrillar collagens such as collagen I, III and V. Type VI collagen (COL6) forms a microfibrillar network that is often associated with type I collagen fibrils and may bind them together to form thicker collagen fibers (5). The COL6 molecule consists of relatively short triple-helical regions about 60 nm long separated by globular domains about 40 nm long. Fibrils of pure COL6 thus give the impression of beads on a string (5). The COL6 protein is comprised of three different alpha chains and the major form is made up of $\alpha 1$, $\alpha 2$, $\alpha 3$ chains. We and others have shown previously that the N-globular domain of the $\alpha 3$ chain of COL6 (COL6A3) consists of a tandem array of 10 homologous protein modules of approximately 200 amino acids long, 9 of which are encoded by a single exon (6–8) (Fig 1). Recent genome exon array studies have identified a tumor-specific alternative splicing of exons 3, 4 and 6 of COL6A3 in colon cancer, and of exon 6 in colon, bladder and prostate cancers (9,10). However, its expression, alternative splicing and function in PDA have not been investigated. In this study, we evaluated the expression and localization of COL6A3 in PDA, intraductal papillary mucinous neoplasms (IPMN) and pancreatic cystadenomas and compared it to the adjacent non-malignant tissue. We also investigated the presence of tumor-specific alternative splicing events in the COL6A3 gene in different tumorigenic and non-tumorigenic human PDA cells lines.

MATERIALS AND METHODS

Human Tissue Acquisition and Analysis

Histologically confirmed human invasive PDA (n=18), premalignant specimens (IPMN adenomas) (n=5) and cystadenomas (n=5, 3 serous and 2 mucinous) were obtained from patients who underwent surgical resection at the Thomas Jefferson University Hospital between 2006 and 2009. All patients signed an appropriate consent for tissue acquisition and study. The study was approved by the Institutional Review Board of Thomas Jefferson University. Tissue samples were stored in RNA Later for RNA analysis or fixed in neutral

formaline for histological processing. Patients' clinicopathological parameters were extracted from the clinical notes and from our Department of Surgery Pancreas database.

Immunohistochemistry

Paraffin blocks were sectioned at 5 μ m. To localize COL6A3, sections from the different tissues were analyzed by immunohistochemistry using a polyclonal antibody raised against the N-terminal domain of COL6A3 (11a). Sections were deparaffinized by treatment with xylene for 5 min (2x) and rehydrated by passage through descending concentrations of alcohol. Antigen retrieval was performed by microwaving the slides in 100 mM sodium citrate buffer for 15 min. Endogenous peroxide activity was quenched by incubating the slides for 5 min in 3% H₂O₂. A vectastain universal elite ABC kit and 3,3'-diaminobenzidine tetrahydrochloride chromogenic substrate (Vector Laboratories Inc.) were used according to the manufacturer's protocol to visualize the tissue reaction. Antibody specificity was validated with nonimmune isotype serum. Negative control sections, where the primary or secondary antibodies were omitted were also prepared. For quantitative assessment of staining of COL6A3, images were captured using a color SPOT camera (Diagnostic instruments, Inc, Sterling Heights, MI) and analyzed using ImagePro plus software. Digital images were captured and color segmentation was performed. Briefly, positive staining area was selected automatically by using a positive staining standard color. The selected area was spectrally separated in red (R), blue (B), green (G) and grey (Gr) and total optical density was calculated through the following formula: $OD = \text{area for positive staining} \times (1/\text{red intensity} + 1/\text{blue intensity} + 1/\text{green intensity} + 1/\text{grey intensity})$. Average optical density for each lesion was compared pairwise. All histological assessment and image analysis was performed on coded, randomized sections by a blinded observer.

Protein isolation and Western blot analysis

The expression of COL6A3 protein was analyzed in 18 PDAs and their matching adjacent tissues. Cell lysates were analyzed as described elsewhere (11). Briefly, protein concentrations in the supernatant were determined using the BCA protein assay reagent (Pierce; Rockford, IL). Equal protein concentrations (40 μ g) were denatured in a gel loading buffer at 85 °C for 5 minutes, loaded onto 3–8 % NuPAGE gels (Invitrogen), transferred to polyvinylidene difluoride membranes, and incubated at 4 °C overnight with the rabbit polyclonal antibody against COL6A3 diluted at 1:5000 in phosphate buffered saline-Tween 20 (PBST) (5). To avoid sample loading errors, β -actin expression was determined in the blots to adjust and normalize the amount of sample loaded (Sigma). The protein bands were visualized with enhanced chemiluminescence reagents (ECL Plus Western Blotting Detection System; Amersham Pharmacia Biotech), analyzed and intensity quantified using Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290).

Cell culture

The human PDA cell lines MIAPACA-2, ASPC-1, BXPc-3 and PANC-1 were purchased from the American Type Culture Collection (Manassas, VA). HS766T and PK9 cell lines were generously donated by Dr. Scott Kern, Johns Hopkins University School of Medicine, Baltimore, MD. HPAF cells were kindly provided by Dr. Surinder Batra, University of Nebraska Medical Center, Omaha, NE. The non-tumorigenic ductal cell line, H6C7, was a kind gift from Dr. Ming-Sound Tsao, University Health Network, Canada. Cells were counted and cultured at 1×10^4 cells to near confluence in 96-well plates and maintained in DMEM supplemented with 10% fetal bovine serum in a humid atmosphere of 5% CO₂/95% air.

Animal studies

Animal studies were performed after approval of the protocols by the IACUC at Thomas Jefferson University. A xenograft model for PDA, using H766T cells was generated in 4 week old male nude mice (CrI:Nu/Nu-muBR) weighing 20–22 g (Charles River laboratories (Wilmington, MA). Mice were injected in their subcutis with a suspension containing 100×10^6 cells/mL in a serum-free media mixed with an equal volume of Matrigel (Collaborative Research), as described previously. Animals were observed daily, and weighed weekly. Tumors were collected after 5 weeks and the expression of COL6A3 in the tissue sections was analyzed by immunohistochemistry. In other studies, we used histological sections from the LSL-KRAS^{G12D} PDX-1-Cre mice at 10 months of age to examine the cellular and stromal expression of COL6A3 by immunohistochemistry.

RNA Extraction and Semi-quantitative PCR

RNAs from cells and whole human tissues were quantified, DNase-digested, and cDNAs were prepared using ImProm-IITM Reverse Transcription System (Promega), then subjected to semi-quantitative PCR using master mix (Promega). The primers used were Exon 3 forward: 5'-AGCAGCAAGCAGATGTCAAA-3', reverse: 5'-TTTCTCCACAGCTAAGGATTT-3'; Exon 4 forward: 5'-ATTGTCTTTGAAACCGCCAAC-3', reverse: 5'-AGCAATGAAGTCTCGGATGG-3'; Exon 6 forward: 5'-TCTCAGGACCTCTCTGGACC-3', reverse: 5'-TAAGGGAAATTGGTTTTTCCAA-3'. Total COL6A3 forward: 5'-TCTCTTAAAATCAGTGCACAACG-3', reverse: 5'-AACTCTTTCAACAGAGGGAAGC-3'. Upstream and downstream primers that could anneal with the 3'-untranslated region of human GAPDH were included in the PCR reaction as an internal standard, forward: 5'-TGAAGGTCTGGAGTCAACGGATTTGGT-3', reverse: 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The linear range of amplification for each set of primers was determined to ensure that we used a number of cycles in the linear range. PCR products were electrophoresed on 2% agarose gels and band intensities were quantified using Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290).

RNA extraction and real-time reverse transcription PCR

Total RNA was isolated from human PDA tissue using Trizol reagent (Life Technologies, Gaithersburg, MD). RNAs were quantified and input amounts were optimized for each amplicon. E6 and GAPDH (internal control) primers and probes were designed with the help of Primer Express Software (Applied Biosystems, Foster City, CA). cDNA was prepared, diluted, and subjected to real-time PCR using the TaqMan technology (7500 Sequence Detector; Applied Biosystems). The relative mRNA levels were presented as unit values of $2^{[C_T(\text{COL6A3-E6}) - C_T(\text{GAPDH})]}$, where C_T is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

Statistical analysis

All experiments were performed 4 to 6 times. Data were analyzed for statistical significance by ANOVA with post-hoc student t test analysis. Data are presented as mean $\pm$ SEM. Continuous, normally distributed variables were analyzed by Student-t-test. Analyses were performed with the assistance of a computer program (JMP 5 Software SAS Campus Drive, Cary, NC). Differences were considered significant at $P \leq 0.05$.

RESULTS

Expression of COL6A3 protein

Patients' data are depicted in table I, including the American Joint Committee on Cancer (AJCC) staging. Protein bands were visualized and their intensity was quantified and average densitometry values were calculated. According to the difference in protein band intensity between malignant and adjacent tissues, expressions was identified as: strong-positive (+++) when band intensity was > 3 fold higher than in non-malignant tissue, moderate-positive (++) when band intensity was 2–3 fold higher, mild-positive (+), when band intensities were 1–2 fold higher, and negative (–) when no bands could be detected (Fig 2).

High levels of COL6A3 were detected in 14 (77%) of the examined 18 cases, of which 9 (50%) were strongly positive. COL6A3-negative tumors were found in only 1 case (6%). Interestingly, the majority of the patients that expressed high levels of COL6A3 presented at later stages (IIB), whereas the ones that presented at an earlier stage expressed low levels of COL6A3 (Table II).

Immunohistochemical expression of COL6A3

As shown in Figure 3A, in the normal pancreas, immunoreactivity of COL6A3 was detected in the connective tissue surrounding the ducts. In IPMN lesions, a more intense expression of COL6A3 could be seen in the stroma with no expression in the transforming ducts (Fig 3B). In PDA, COL6A3 was highly deposited mainly in the desmoplastic stroma around the malignant ducts (Fig 3C) and in between the stromal fatty infiltrations (Fig 3D). These data indicate that a COL6A3 is constitutively expressed in the malignant stroma. Figure 3E shows quantification of stromal COL6A3 staining density in normal, IPMN and PDA lesions. IPMN show higher immunoreactivity than the normal ducts and the difference in PDA was highly significant.

COL6A3 isoform expression

Using specific primers for exons 3, 4 and 6 of COL6A3 and GAPDH as internal control, we analyzed by semi quantitative PCR for their presence in IPMN (n=5), cystadenomas (n=5) and PDA and matched adjacent tissue (n=18). As seen in Fig 4A, the 3 isoforms were absent from all the IPMN lesions and the cystadenomas. In contrast, all PDA lesions showed inclusion of the 3 exons with variable levels in the adjacent tissues (Fig 4B). Exon 4 and to a lesser extent, exon3 were exclusively present in the malignant lesions. Since the semi quantitative PCR results for exon 6 did not show a consistent difference between PDA and their matched adjacent tissue, we analyzed the tissue by real time PCR using unique primers that amplify exon 6. As seen in Fig 4C, variable levels of exon6 were expressed in the different lesions with consistent upregulation of exon6 in PDA when compared to the adjacent tissue. These data show for the first time the exclusive presence of COL6A3 isoforms in PDA and their absence from the premalignant lesions.

COL6A3 isoform expression in human PDA cell lines

Using semi quantitative PCR, we investigated whether human PDA cells express COL6A3 or its isoforms. As seen in Fig 5A, all PDA cell lines expressed total COL6A3 mRNA. All cell lines expressed E6 except for the non-tumorigenic cell line H6C7. Interestingly, ASPC-1 and MIAPACA-2 cell lines showed inclusion of both E3 and E4 isoforms, which correlated with high total COL6A3 mRNA and E6. We then analyzed the expression of COL6A3 in the immortalized human fibroblast cell line, hTERT-BJ1 (Clontech, Inc). Total COL6A3 and its 3 isoforms were clearly seen to be expressed in this cell line (Fig 5B).

These data indicate that in addition to fibroblasts, some PDA cells could be a source of COL6A3 isoforms.

Expression of COL6A3 in animal models of human PDA

Next we sought to detect the source(s) of COL6A3 *in vivo*. Immunohistochemical analysis of PDA xenografts revealed the presence of COL6A3 protein in the connective tissue between the tumor cells (Fig 6A). Higher magnification revealed that COL6A3 is present in giant multinucleated cancer cells and spindle-shaped fibroblasts (Fig 6B). Analysis of tissue sections from the LSL-KRAS^{G12D};PDX-1-Cre mice, which develop PanIN lesions that progress eventually to invasive pancreatic cancers (12), showed the deposition of COL6A3 in the connective tissue, in fibroblasts and in a population of transforming ductal cells (Fig 6C). Our data suggest that malignancy in PDA is associated with a dynamic process of alternative splicing of COL6A3, which results in increased mRNA and protein expression levels. Our data also suggest that, in addition to cancer associated fibroblasts, a certain population of tumor cells may be actively producing COL6A3.

DISCUSSION

The results of this study demonstrate for the first time that the malignant pancreatic stroma and certain PDA cells exclusively express several COL6A3 isoforms and suggest that these isoforms might serve a critical function during the process of malignant transformation.

Previous genetic studies of PDA have focused mainly on the molecular aberrations in the transforming ductal epithelium. Ductal cells overexpress many mitogenic growth factors and their corresponding tyrosine kinase receptors (13). They also have a high frequency of mutations in the K-ras oncogene, the p53 and Smad4 tumor suppressor genes and the p16 cell cycle inhibitory gene (14). There is also excessive activation of downstream signaling pathways, such as src, nuclear factor κ B and Stat3 (15–17), which facilitate tumor cell proliferation, suppress pro-apoptotic pathways and promote the spread and metastasis of tumors (18). However, very few studies have looked at the structure of PDA stroma and the role it plays in cancer development and progression in PDA.

Collagen VI has been reported to be increased in the tumor microenvironment and has been hypothesized to be important for extra cellular matrix- (ECM)-tumor interactions (19,20). In addition, the presence of ECM components and related signaling can reduce cell susceptibility to apoptosis and might be responsible for increased chemotherapeutic drug resistance in small cell lung cancer (20, 21). In our study, we show that the total levels of COL6A3 protein are highly upregulated. We show here that the stroma of malignant PDA express high levels of COL6A3 that correlated with more advanced stage (Table II)

COL6A3 gene encodes a chain of collagen VI, a microfibrillar collagen that contains subdomains similar to the type A domains of the von Willebrand factor, fibronectin type III domains, and a C-terminal Kunitz-type module (22). Collagen VI is believed to be involved in cell anchoring as well as signaling through interactions with integrins (23) and possibly other receptors such as NG2 (24) and DDR1/2 (25). Collagen VI has been shown to bind to several proteins of the ECM, including decorin (26), an interaction that was suggested to increase cancer resistance to chemotherapy (27).

Recent genome exon array studies have identified a unique expression of tumor-specific alternative splicing of exon 6 of COL6A3 in colon, bladder and prostate cancers, and exons 3 and 4 in colon cancer (9,10). In our studies, we identified for the first time exons 3, 4 and 6 as exclusive to PDA (Fig 4B). Although semi-quantitative PCR showed that exon 6 seemed to be equally expressed in several malignant and adjacent tissue pairs (Fig. 4B), real

time PCR data showed that it is highly and consistently upregulated in the tumor tissue (Fig 4C). Furthermore, the three exons (3,4, and 6) were absent in the benign lesions (Fig 4A), suggesting that inclusion of these exons may contribute to the etiology or progression of malignancy in PDA. Moreover, the specificity of the exons to the tumor tissues suggests their potential to serve as molecular biomarkers for the diagnosis or prognostic prediction of PDA.

We show in our immunohistochemical analysis of human paraffin sections that COL6A3 is highly deposited in between the malignant ducts (Fig 3C, D). However, we could not detect it in the malignant epithelial cells. Thus, we evaluated several PDA cell lines for the presence of COL6A3 and for the presence of alternative splicing events. We show that all PDA cells express total COL6A3 and exon 6, except for the non tumorigenic cell line H6C7 (Fig 5A). In addition, the immortalized fibroblasts (hTERT-BJ1) expressed all three isoforms (Fig 5B). It is unknown whether interactions between tumor cells and fibroblasts in the stroma are responsible for producing more COL6A3 in the desmoplastic stroma. Interestingly, the 2 cell lines that expressed exons 3 and 4 (ASPC-1 and MIAPACA-2) also expressed elevated levels of total COL6A3 mRNA, suggesting that the presence of exons 3 and 4 maybe responsible for elevating the gene expression levels. It is yet to be determined, however, whether the presence of these isoforms correlates with increase in the carcinogenic behavior of cancer cells. Studies in this regard are currently ongoing in our lab.

Further analysis of PDA xenografts in vivo revealed the presence of the COL6A3 protein in giant multinucleated cancer cells and spindle-shaped fibroblasts (Fig 6A). We then investigated the cellular source of COL6A3 in the LSL-KRAS^{G12D};PDX-1-Cre mice. This mouse model recapitulates the premalignant state, and grade I and II pancreatic intraepithelial neoplasia (PanINs) are associated with a small amount of normal stroma surrounding the normal pancreatic ducts from which PanINs arise. When these lesions progress to PanIN III lesions, there is usually enhanced stromal formation, while advancement to invasive carcinoma often is associated with extensive stroma formation (28, 29). The majority of this stromal fibrosis is composed of fibrillar collagens (collagen types I, III and V) which are secreted by proliferating fibroblasts and pancreatic stellate cells (30). Recent studies have linked isoforms of collagen I to invasive behavior in carcinomas, which suggests that the stroma plays a role in tumor progression through alternative splicing (31). Analysis of tissue sections from the LSL-KRAS^{G12D};PDX-1-Cre mice show the deposition of COL6A3 in the connective tissue, in fibroblasts and in a population of transforming ductal cells (Fig 6C), suggesting that, in addition to cancer associated fibroblasts, a certain population of tumor cells may be actively producing COL6A3.

In conclusion, alternative splicing is a mechanism for increasing protein diversity that may serve as a means for cancer diagnosis. In this study, we show that a dynamic process of alternative splicing of the COL6A3 gene is exclusively associated with malignancy in PDA. Inclusion of exons 3, 4, and 6 correlates with increased total COL6A3 mRNA and protein levels. In this regard, it is particularly interesting that the COL6A3 isoform presence and high protein levels appear to correlate with tumor stage. Further studies to evaluate the oncogenic function, the diagnostic potential and the ability to predict prognosis of the individual COL6A3 isoforms are currently ongoing.

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Figure 1.

Schematic diagram of the three collagen VI chains (COL6A1, COL6A2 and COL6A3). Each chain contains a triple helical domain (TH) of 335–336 amino acids, which is flanked by N- and C-globular domains consisting of vWF-A modules (~200 amino acids/module), designated N1–N10 and C1–C2. The N10, N9 and N7 subdomains are either present or absent in the final COL6A3 chain due to alternative splicing of exons 3, 4, and 6 (E3, E4, E6).

Figure 2.

Representative Western immunoblots of protein extracts from four PDA and adjacent tissues showing expression of COL6A3. Average densitometry values of the samples were multiplied to obtain the arbitrary levels. (+++) = strong-positive band intensities that are > 3 fold higher than in adjacent tissue, (++)= moderate-positive band intensities that are 2–3 fold higher, (+) = mild-positive band intensities that are 1–2 fold higher than the non malignant tissue and (–) no expression of COL6A3.

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 3E

Figure 3.

Representative immunohistochemical staining of paraffin embedded pancreatic sections stained with COL6A3. **A.** low COL6A3 immunoreactivity in the adjacent non malignant tissue **B.** Higher periductal immunoreactivity of COL6A3 in IPMN. In PDA, high immunoreactivity is seen mainly in the desmoplastic stroma (**C**) and in between the stromal fatty infiltrations (**D**). Negative control (Neg C) samples where the primary antibody was omitted did not show non-specific reaction. X 100 original magnification. **E.** Three randomly selected images from each lesion section were digitized. By means of a predefined positive staining color standard, positively stained areas were marked automatically. Values of the total positive staining area (pixel), distribution and magnitude of the color intensity in four different channels were obtained and the total optical density was calculated in logs of optical density as described under materials and methods.

Fig 4A

Fig 4B

Fig 4C

Figure 4.

A. Representative RT-PCR from 3 IPMN and 3 cystadenoma lesions showing absence of the three alternatively spliced exons. **B.** Representative RT-PCR from 8 PDA tumors (T1–T8) and adjacent tissues (A1–A8) showing expression of exons 4, 3 and 6 mainly in the tumors with lower or absent expression in the surrounding tissue. **C.** Real time PCR for E6 shows differential expression levels between individual tumors and consistently higher levels in the tumor tissue when compared to the surrounding adjacent tissues.

Fig 5A

Fig 5B

Figure 5.

A. Representative of RT-PCR showing that, with the exception of two cell lines, AsPC-1 and MIAPACA-2 that express high levels of COL6A3 and E3, E4, and E6, most PDA cells express low levels of COL6A3 and E6. The non-tumorigenic H6C7 cells did not express COL6A3 isoforms. **B.** Representative of RT-PCR showing immortalized human skin fibroblasts (hTERT-BJ1) express COL6A3 and its three splice variants. M = marker

Figure 6A

Figure 6B

Figure 6C

Figure 6.

Representative of 5 μ m paraffin section of xenografts of Hs766T cells immunostained with COL6A3 antibody. **A.** COL6A3 could be localized mainly to the connective tissue stroma (upper panel X 100 original magnification). Most of the malignant cells did not express the protein in vivo (lower panel X200 original magnification). **B.** Higher magnification (Fig 1C X400 original magnification) identified two types of cells that express COL6A3. Large multinucleated cells that are usually localized near the interstitial spaces (black arrows), and spindle shaped fibroblasts (red arrows). **C.** Representative pancreatic histologies of LSL-KRAS^{G12D};PDX-1-Cre mice at 10 months of age. left, dilated malignant duct with luminal detached ductal cells; Middle, high magnification; Right, higher magnification showing

expression of COL6A3 in fibroblasts (red arrows), the CT surrounding the ducts and in some population of ductal cells (black arrows)

Table I

PDA patients included in this study. *n*, number of patients; PDA, pancreatic ductal adenocarcinoma; *IIA*, tumor growing outside the pancreas, no lymph node or distant metastasis; *IIB*, tumor growing outside pancreas, lymph node metastasis, no distant metastasis; *IV*, distant metastasis present. *NI*, non-invasive.

PDA Patients (total number=18)		
	<i>n</i>	Percentage
Gender		
Men	8	44
Women	10	56
Median Age (years)		
Men	64.7	
Women	66	
IIA (NI)	2	11
IIB	14	78
IV	2	11

Table II

Analysis of protein band density values of COL6A3/actin. The majority of the patients that expressed high levels of COL6A3 presented at later stages (IIB), whereas the ones that presented at an earlier stage expressed low levels of COL6A3.

Patients (total number=18)			
	<i>n</i>	Percentage	AJCC Stage
COL6A3 (+++)	9	50	8: IIB, 1:IV
COL6A3 (++)	5	28	1: IV, 1: IIA, 3:IIB
COL6A3 (+)	2	11	2: IIB
COL6A3 (–)	2	11	1: IIA, 1 :IIB