Published in final edited form as:

Curr Mol Med. 2012 March ; 12(3): 331–341.

Genomic Sequencing of Key Genes in Mouse Pancreatic Cancer Cells

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Abstract

Pancreatic cancer is a multiple genetic disorder with many mutations identified during the progression. Two mouse pancreatic cancer cell lines were established which showed different phenotype *in vivo*: a non-metastatic cell line, Panc02, and a highly metastatic cell line, Panc02-H7, a derivative of Panc02. In order to investigate whether the genetic mutations of key genes in pancreatic cancer such as *KRAS*, *TP53* (*p53*), *CDKN2A* (*p16*), *SMAD4*, *ZIP4*, and *PDX-1* contribute to the phenotypic difference of these two mouse pancreatic cancer cells, we sequenced the exonic regions of these key genes in both cell lines and in the normal syngeneic mouse pancreas and compared them with the reference mouse genome sequence. The exons of *KRAS*, *SMAD4*, *CDKN2A* (*p16*), *TP53* (*p53*), *ZIP4*, and *PDX-1* genes were amplified and the genotype of these genes was determined by Sanger sequencing. The sequences were analyzed with Sequencher software. A mutation in *SMAD4* was identified in both cell lines. This homozygote G to T mutation in the first position of codon 174 (GAA) generated a stop codon resulting in the translation of a truncated protein. Further functional analysis indicates that different TGF- / SMAD signaling pathways were involved in those two mouse cell lines, which may explain the phenotypic difference between the two cells. A single nucleotide polymorphism (SNP) in *KRAS* gene (TAT to TAC at codon 32) was also identified in the normal pancreas DNA of the syngenic mouse and in both derived tumoral Panc02 and Panc02-H7 cells. No mutation or SNP was found in *CDKN2A* (*p16*), *TP53* (*p53*), *ZIP4*, and *PDX-1* genes in these two cell lines. The absence of mutations in genes such as *KRAS*, *TP53*, and *CDKN2A*, which are considered as key genes in the development of human pancreatic cancer suggests that *SMAD4* might play a central and decisive role in mouse pancreatic cancer. These results also suggest that other mechanisms are involved in

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

the substantial phenotypic difference between these two mouse pancreatic cancer cell lines. Further studies are warranted to elucidate the molecular pathways that lead to the aggressive metastatic potential of Panc02-H7.

Keywords

CDKN2A (p16); genomic sequence; *KRAS*; mouse pancreatic cancer; *PDX-1*; *SMAD4*; *TP53 (p53)*; *ZIP4*

INTRODUCTION

Pancreatic cancer is an extremely malignant disease associated with poor prognosis. The 5-year survival rate is less than 5% in pancreatic cancer patients. Although moderate progress in diagnosis and therapy has been made, the statistics remain unchanged for the past several years. In 2010, it was estimated that 44,030 patients were diagnosed with pancreatic cancer in the U.S, and among these patients, 37,660 patients had died [1]. Currently, surgical resection is the only curative treatment. Unfortunately, only about 10–15% of patients are eligible for surgical resection and cancer recurrences for these patients are common [2]. Consequently there is an urgent need for developing novel methods for early diagnosis and therapy of pancreatic cancer.

A number of mutations in key genes such as *KRAS*, *SMAD4*, *CDKN2A (p16)*, and *TP53 (p53)* have been identified in human pancreatic cancer [3]. Discoveries of these mutated genes have provided important insight to the development of pancreatic cancer and suggested new diagnostic and therapeutic strategies for the patients. Recent studies have identified new molecular targets in pancreatic cancer, whose overexpression promote pancreatic cancer progression, such as *ZIP4* and *PDX-1* genes. Our previous studies indicated that *ZIP4* is upregulated in human pancreatic cancer, and silencing of *ZIP4* led to decreased pancreatic cancer growth and increased survival rate in a xenograft mouse model [4, 5]. *PDX-1* has been shown to exert oncogenic properties in pancreatic cancer [6]. Those data strongly suggest that *ZIP4* and *PDX-1* are novel molecular targets in pancreatic cancer and might serve as cancer master switch genes in pancreatic cancer development and progression. Therefore, detailed genetic profiling study on those above mentioned key genes in pancreatic cancer is of great interest in elucidating the molecular mechanisms of this deadly disease.

Animal models, especially mouse models, are often employed for studying the genetic variations and the biological relevance in cancer progression. Panc02 and its metastatic derivative Panc02-H7 cell lines were established to study the pancreatic cancer genetics and immunology in a syngeneic mouse model. After implantations of these cells in the pancreas, the highly proliferating Panc02-H7 cells disseminated to peritoneum and metastasized to distal organs, showing more aggressive invasion compared to the parental Panc02 cells [7]. These two mouse pancreatic cancer cells are important tools to study the immune response against pancreatic cancer in immune competent mice and to test new immunotherapy drugs and vaccines. Therefore, it is critical to examine the genomic profile of key genes in human pancreatic cancer such as *KRAS*, *SMAD4*, *CDKN2A (p16)*, *TP53 (p53)*, *ZIP4*, and *PDX-1* in these mouse pancreatic cancer cell lines. Through comprehensive analysis we can then compare their genomic sequences in order to pinpoint the driver mutations, genetic variations, or other defects in the key signaling pathways which give rise to the substantial biological differences of those cells.

MATERIALS AND METHODS

Chemicals and Reagents

DMEM medium and fetal bovine serum were purchased from Thermo Fisher Scientific Inc. (Logan, UT). The QIAamp DNA Mini Kit and QIAquick Gel Extraction Kit were purchased from QIAGEN Inc. (Valencia, CA). FastStart Taq DNA Polymerase and dNTPPack were purchased from Roche Inc. (Indianapolis, IN). Agarose was purchased from SIGMA Co. (St. Louis, MO).

Cell Line and Cell Culture Condition

Mouse pancreatic cancer cell line Panc02 was originally established by Corbett *et al.* [8]. The highly metastatic Panc02-H7 cell line was established using an *in vivo* selection method by Wang *et al.* [7]. These cells were cultured in DMEM medium with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂.

DNA Extraction

Genomic DNA was extracted from the pancreas of a C57BL/6 mouse and Panc02 and Panc02-H7 derived cell lines using QIAamp DNA Mini Kit in accordance with the manufacturer's instructions. In brief, 25 mg of mouse tissue in 80 µl phosphate buffered saline (PBS) was homogenized using a Bullet Blender (Next Advance, Inc). 100 µl Buffer ATL and 20 µl proteinase K were added and incubated at 56°C for 2 hours until the tissue is completely lysed. 200 µl Buffer AL was added to the sample and incubated at 70°C for 10 min. 200 µl 100% ethanol was added to the sample and transferred to the QIAamp Mini spin column. The spin column was washed twice and the DNA was eluted with 200 µl of distilled water. Extracting genomic DNA from cells is as follows: cells at 80% confluence were harvested with a cell scraper and washed with PBS. The cell pellet was suspended in PBS to a final volume of 200 µl. 20 µl of proteinase K and 200 µl of buffer AL were added, and the solution was mixed by pulse-vortexing for 15 s before incubated at 56°C for 10 min. After centrifugation, 200 µl of 100% ethanol was added to the supernatant and the mixture was transferred to a QIAamp Mini spin column. The spin column was washed twice and the DNA was eluted with 200 µl of distilled water. The DNA concentration was measured by BioTek Gen5 data analysis software.

Primer Design

In order to identify the genetic variations and the driver mutations in key genes of mouse pancreatic cancer cells, multiple primer sets were designed to cover the coding regions of six genes including *KRAS*, *SMAD4*, *CDKN2A (p16)*, *TP53 (p53)*, *ZIP4*, and *PDX-1* (Supplementary table). They were designed using a serial linked pipeline as described previously to ensure the high fidelity of the sequencing results [9]. All primers were validated to meet quality control. Universal sequencing primers were added in both ends for the ease of subsequent Sanger sequencing. The universal primer sequences are as follows: Forward: "5'CTCGTGTAACGACGGCCAGT3'", Reverse: "5'CTGCTCAGGAACAGCTATGAC3'". As shown in Fig. (1), the PCR products covered all the coding sequences of the six genes. For the larger exons that cannot be covered by a single pair of primers, two or three pairs of primers were used so that the entire coding sequences were completely covered.

Polymerase Chain Reaction (PCR)

The PCR mixture (50 µl) consisted of 200 ng of DNA template and 1 µl of each forward and reverse primer (10 µM). PCR amplification was programmed with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation, annealing and primer

extension. The optimal annealing temperature depending on the primer sequences usually ranges between 58°C to 62°C. The annealing cycle was set to 30 s each. The condition of primer extension step was 72°C for 30 s per cycle. Upon completion of the cycling steps, a final extension step was performed at 72°C for 7 min and the sample was stored at 4°C.

PCR Products Purification and Sequencing

The DNA was electrophoresed in a 1% Agarose gel and the PCR products were cut out of the gel. Then, the PCR products were purified using QIAquick Gel Extraction Kit from QIAGEN according to the manufacturer's recommendations. In brief, 3 volumes Buffer QG to 1 volume gel were added and the sample was incubated at 50°C for 10 min. One gel volume of isopropanol was added to the sample which was then transferred to a QIAquick spin column placed in a clean 2 ml collection tube. The spin column was centrifuged at 14,000 rpm for 1 min and the buffer PE was added to wash the sample for 2–5 min. The washed sample was then centrifuged for 1 min. The spin column was centrifuged once again and the sample was transferred a new collection tube. To elute the sample, 30 µl distilled water was added and the sample was incubated at room temperature for 1 min. After incubation, the sample was centrifuged at 14,000 rpm for 2 min and the concentration of PCR product was determined. The PCR products were sequenced by Sanger sequencing using the universal sequencing primers.

Sequence Analysis

Sequences from the normal syngeneic C57BL/6 mouse pancreas, the derived tumoral cell lines were aligned and compared among themselves, and to the mouse reference genome (NCBI) using the sequence alignment software Sequencher v4.7 (Gene Codes Co, Ann Arbor, MI).

Western Blot Analysis

Panc02, Panc02-H7, and Panc-1 cells were lysed with ice-cold lysis buffer (1X NuPAGE LDS Sample Buffer, Invitrogen) for 5 min on ice. Cell lysates were then collected after centrifugation at 13,000 rpm for 5 min at 4°C. Sixty µg of lysate protein was loaded and total cellular protein was separated with 12% SDS-polyacrylamide gel electrophoresis and then transblotted overnight at 4°C onto Hybond-P PVDF membrane (Amersham Biosciences). The membrane was probed with anti-SMAD4 (1:1000), or anti- β -actin (1:10,000) antibodies at 4°C for overnight and then washed three times with 0.1% Tween 20-TBS and incubated in a horseradish peroxidase-linked secondary antibody (1:2000) for 1 h at room temperature. The membrane was washed three times with 0.1% Tween 20-TBS and the immunoreactive bands were detected by using enhanced chemiluminescent (ECL) plus reagent kit.

Site-Directed Mutagenesis

Using the expression vector pcDNA3.1-SMAD4-WT as template, the mutant SMAD4-m174 was constructed with mutation-specific primers and QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent). The mutagenesis primers are: Forward: "5'AGCCGTCCTTACCCA CTTAAGGACATTCGATTCAA3'", Reverse: "5'TTGAA TCGAATGTCCTTAAGTGGGTAAGGACGGCT3'". The presence of the mutation was confirmed by sequencing analysis.

Dual-Luciferase Reporter Assays

Panc02, Panc02-H7, and Panc-1 cells were transiently transfected with TGF- β -inducible reporter vector 3TP-luciferase reporter vector (3TP-lux) and control vector RL-TK, as well as pcDNA3.1, pcDNA3.1-SMAD4-m174 or pcDNA3.1-SMAD4-WT in 24-well plates,

respectively, using Lipofectamine™ 2000 (Invitrogen, CA). 24 hrs after transfection, cells were treated with or without 10ng/ml TGF- β in DMEM containing 0.2% FBS for another 12 hrs. Cells were then lysed and luciferase activities were measured with a luciferase plate reader (Synergy HT, BioTek) using the dual luciferase assay kit (Promega, Madison, WI). All luciferase activity was normalized to an internal renilla luciferase transfection control RL-TK.

Statistical Analysis

Quantitative results are shown as means $\pm$ standard deviations. The statistical analysis was performed by the Student's t test between control and treatment groups. A p-value of < 0.05 was considered statistically significant.

RESULTS

KRAS Gene Has a Single Nucleotide Polymorphism (SNP) in Two Mouse Pancreatic Cancer Cell Lines

In human, *KRAS* mutation appears in the early stage of pancreatic cancer development with an incidence of 75%-100% at codon 12. The most frequent base shifts at codon 12 are: GGT to GAT (46%), GTT (32%) and CGT (13%) [10]. No mutation of *KRAS* in mouse pancreatic cancer cells was reported. We found a synonymous polymorphism in the normal pancreas of C57BL/6 mouse, Panc02, and Panc02-H7 cells in codon 32 which led to a TAT to TAC coding sequence change without any amino acid sequence change (Table 1 and Fig. 2). No other SNP or mutation was found in the coding sequences in the two tumor cell lines.

A Novel Non-Synonymous Mutation in *SMAD4* Gene Was Identified in Two Mouse Pancreatic Cancer Cell Lines

The high frequency of *SMAD4* mutations is associated with poor prognosis of pancreatic cancer patients [11]. Many different *SMAD4* mutations in human pancreatic cancer were reported. Examples of *SMAD4* mutations include transversion of GGA to TGA at codon 358 (Gly to stop), transversion of TAC to TAG at codon 412 (Tyr to stop), and transversion of GAT to CAT at codon 493 (His to Asp) [12] (Table 1). No SNP was detected in the normal pancreas of C57BL/6 mouse. However, we found a non-synonymous *SMAD4* mutation GAA to TAA (Glu to stop) at codon 174 in both mouse cell lines. Since a single base was detected at the mutation location, this most likely is the result of a mutation in one of the allele and a deletion of the region in the second allele or less probably the presence of the same mutation in both alleles. Both cases result in a truncated protein (Fig. 3 and Table 1). This mutation probably changes the protein structure and function in mouse pancreatic cancer cells.

No Mutation in *CDKN2A* (p16), *TP53* (p53), *ZIP4*, and *PDX-1* Genes was Found in Mouse Pancreatic Cancer Cells

As shown in Table 1, diverse mutations were reported in human for *CDKN2A* (p16), *TP53* (p53), *ZIP4*, and *PDX-1*. *CDKN2A* (p16) is another frequently mutated gene in pancreatic cancer with reported mutations as follows: G23D, R80stop and H83Y [13]. The mutation of the tumor suppressor gene *TP53* (p53) is also found in pancreatic cancer which is responsible for late-stage PanIN progression. Some of the *TP53* (p53) mutation hotspots are R175H, R248Q and R273H [14]. Although the incidence of *CDKN2A* (p16) and *TP53* (p53) mutation is high in pancreatic cancer patients and many human pancreatic cancer cell lines, we did not find any mutation of *CDKN2A* (p16) and *TP53* (p53) in these two mouse pancreatic cancer cell lines (Table 1 and Fig. 4). Aberrant *ZIP4* expression was found to promote pancreatic cancer growth [4]. But currently there is no mutation of *ZIP4* gene

reported in pancreatic cancer. The rare *ZIP4* mutations were found in patients with acrodermatitis enteropathica. Examples of *ZIP4* mutation in acrodermatitis enteropathica patients are: TTA to TGA at codon 48, TGT to CGT at codon 62 and CCG to CTG at codon 84 [15, 16]. The expression of PDX-1 appears aberrant in pancreatic cancer and precursor lesions (PanIN, IPMN and MCN). A few PDX-1 mutations found in type 2 Diabetes Mellitus include TGC to CGC at codon 18, CGC to CAC at codon 197 and CAG to CTG at codon 59 [17,18]. In agreement with human study, there were no mutations for both *ZIP4* and *PDX-1* genes in the two mouse pancreatic cancer cell lines (Table 1 and Fig. 4).

Functional Analysis of the Novel SMAD4 Mutation

To study the function of the mutant SMAD4 in the mouse pancreatic cancer cells, we first examined the expression of SMAD4 protein in Panc02 and Panc02-H7 cells, and included a SMAD4 wild type pancreatic cancer cell line, Panc-1 cells. As shown in Fig. (5), a specific SMAD4 band was clearly seen in Panc-1 cells, but no band of full length SMAD4 was observed in Panc02 and Panc02-H7 cells. Real-time PCR result showed that the endogenous SMAD4 mRNA were present in both cell lines (data not shown), suggesting that the mutation might lead to a truncated protein which is not recognized by the SMAD4 Ab.

We then tested the function of this mutant SMAD4 in TGF- β induced SMAD4 dependent signaling pathway by using a 3TP-lux reporter assay. Panc-1 cells responded to TGF- β , and the transfection of either mutant SMAD4-m174 or wild type SMAD4 did not impact the response to TGF- β in Panc-1 cells (Fig. 6A). Panc02 cells alone or Panc02 cells with the overexpression of the mutant SMAD4-m174 did not respond to TGF- β stimulation, while the response to TGF- β was rescued when the wild type SMAD4 was introduced into the Panc02 cells (Fig. 6B). However, Panc02-H7 cells did not respond to TGF- β , and introduction of wild type SMAD4 did not rescue the response either (data not shown), suggesting that this truncated mutant SMAD4-m174 identified in Panc02 and Panc02-H7 cells lost its normal functions in the TGF- β induced SMAD4 dependent signaling pathway, and additional defects in the TGF- β /SMAD pathway may also exist in Panc02-H7 cells, which might explain the phenotypic difference of these two cells.

DISCUSSION

The current study represents a detailed analysis of the genomic profiling of six key genes in mouse pancreatic cancer. Panc02 cell line was developed by implanting cotton thread-carrying 3-methylcholanthrene into the pancreas of C57BL/6 mouse [8]. Panc02-H7 cell line was derived from Panc02 and shows more aggressive phenotype *in vivo* compared to the parental Panc02 cells. Syngeneic mouse model, with higher tumor take rate and mild inflammatory reactions, is a more appropriate model compared to xenografts to study cancer genetics and immunology. Therefore, Panc02 and Panc02-H7 cells are important tools for syngeneic mouse models of pancreatic cancer, especially for immunotherapy of pancreatic cancer. Further understanding of the genetic background for these two mouse pancreatic cancer cells is essential to develop new therapeutic strategies. Novel genetic alterations in *KRAS* and *SMAD4* genes were identified in the two mouse pancreatic cancer cell lines. Different TGF- β /SMAD signaling pathway defects were suggested in those two mouse cell lines, which may explain the phenotypic difference between the two cells. Conversely, the previously reported mutations with higher incidence in human pancreatic cancer were not found in the six genes of the mouse cell lines.

The *KRAS* gene encodes the guanosine triphosphate (GTP)-binding protein which acts as a switch to transmit downstream signals for cell proliferation, differentiation and apoptosis [19]. Activated *KRAS* mutations are the most frequent genetic events found in pancreatic cancer. Moore, Sipos *et al.* [20] analyzed the genetic profile of 22 human pancreatic cancer

cell lines for alteration in *KRAS* gene and found *KRAS* variants in 20 cell lines. More importantly, *KRAS* mutations were observed in the majority of human pancreatic cancer patients, which is believed to be the first event implicated in the development of pancreatic cancer [10]. For quite a few years, the implications of *KRAS* mutations have been studied in personalized medicine. Recent progress in clinical trials demonstrates how *KRAS* mutations can be successfully incorporated into personalized medicine [21]. Testing *KRAS* mutations can help predict the response to anti-EGFR monoclonal antibody therapy in metastatic colon cancer, which had been approved by FDA [21]. Current experimental data from clinical trials indicates that *KRAS* gene mutations play a vital role in the carcinogenesis of pancreatic cancer [22, 23]. The most frequent point mutations of *KRAS* gene are at codon 12: GGT to GAT (46%), GGT to GTT (32%) and GGT to CGT (13%) [10]. Another point mutation of *KRAS* involves a change from GGC to GAC at codon 13 [24]. In rare cases, a mutation (CAA to CAT) occurs at codon 61 [25]. However, the frequently found *KRAS* mutations in human were not observed in our current study of the mouse pancreatic cancer cells. Instead, a silent SNP at codon 32 was found in both Panc02 and Panc02-H7 cells. Since this is a synonymous SNP, it does not affect the protein expression.

The cancer suppressor gene *SMAD4* (also known as *DPC4*) belongs to the SMAD family and is a critical mediator of TGF- β signaling pathway that suppresses epithelial cell growth [26–28]. Homozygous deletion was observed in approximately 30% of pancreatic cancer [12, 29]. Blackford *et al.* [11] illustrated that somatic genetic alteration of *SMAD4* is associated with the outcome of pancreatic cancer patients who have undergone pancreatic tumor resection. Pancreatic cancer patients with *SMAD4* inactivating mutations had a significantly worse survival curve than patients with wild type *SMAD4*. In our current study, both Panc02 and Panc02-H7 cell lines had a mutation at codon 174, which was not found in wild type C57BL/6 mouse, leading to the expression of a truncated protein. However, no *SMAD4* mutation was observed in the mouse pancreatic cancer cell lines at any of the sites where previous mutations were reported in human pancreatic cancer. *SMAD4* gene mutations are mostly found in pancreatic cancer and colorectal cancer [29]. Moreover, the aberrant TGF- β /SMAD4 signaling, where SMAD4 plays a key role, is critical in the progression of various cancers, such as ovarian cancer and thyroid cancer [30, 31].

SMAD4 protein contains three distinct domains, MH1, MH2 and one linker region which connects the MH1 and MH2 domains. The main function of MH1 domain is to bind to the DNA and MH2 domain is involved in protein-protein interaction. Most of the SMAD4 mutations are found in the MH2 domain [32, 33]. The novel SMAD4 mutation that we discovered in the mouse Panc02 and Panc02-H7 cells is located in the linker region, leading to the early termination of the translation and a truncated SMAD4 (SMAD4-m174) protein, which completely lost the MH2 domain. To validate the function of the mutant SMAD4-m174, we first examined the expression of SMAD4 protein in Panc02, Panc02-H7, and a SMAD4 wild type cell line, Panc-1 cells. Full length SMAD4 was detected only in Panc-1 cells, but not in Panc02 and Panc02-H7 cells, although real-time PCR result showed that the endogenous SMAD4 mRNA were present in these two cell lines. Those data suggest that the mutation might lead to a truncated SMAD4 protein in the mouse pancreatic cancer cells. Further functional studies indicate that compared to the Panc-1 cells, which responded to TGF- β stimulation, Panc02 cells alone or Panc02 cells with the overexpression of the mutant SMAD4-m174 did not respond to TGF- β , while the response to TGF- β was rescued when the wild type SMAD4 was introduced into the Panc02 cells. However, Panc02-H7 cells did not respond to TGF- β , and introduction of wild type SMAD4 did not rescue the response either. Further studies indicate that the SMAD2/3 pathway was also impaired in Panc02-H7 cells compared to Panc02 and Panc-1 cells upon TGF- β treatment (data not shown). Those data suggest that the truncated mutant SMAD4-m174 identified in Panc02 and Panc02-H7 cells lost its normal functions in the TGF- β induced SMAD4 dependent signaling pathway,

and additional defects in the TGF- β /SMAD pathway may also exist in Panc02-H7 cells, which might explain the phenotypic and biological difference of these two cells, and indicate the critical role of TGF- β /SMAD pathway in pancreatic cancer metastasis.

The cancer suppressor gene *CDKN2A*, which is also known as *p16* or *INK4A*, encodes a protein that modulates two important pathways, the *p53* pathway and the retinoblastoma (pRb) gene pathway [34]. Mutations in *CDKN2A* are most prevalent in pancreatic cancer patients (90%) [35]. McWilliams *et al.* [36] found that tobacco use might increase the risk of pancreatic cancer for people with *CDKN2A* mutation. *TP53* (*p53*) is triggered by various deregulated signaling pathways and regulates many pathways involved in tumor formation, invasion and metastasis. Inactivating mutations of *TP53* (*p53*) gene play a pivotal role in tumorigenesis of pancreatic cancer [14] and are observed in about 70% pancreatic cancer [37]. In spite of the high mutation incidence of *CDKN2A* (*p16*) and *TP53* (*p53*) genes in human pancreatic cancer, we did not find any mutation of these two genes in the mouse pancreatic cancer cells. *ZIP4* is a zinc transporter protein involved in intracellular zinc homeostasis, and plays an important role in pancreatic cancer pathogenesis and progression [4, 5]. Currently there is no mutation of *ZIP4* gene reported in human pancreatic cancer. There have been no mutations of *PDX-1* in the exonic DNA to date [38], although at least two mutations in other regions of the *PDX-1* DNA have been confirmed (data not shown). *PDX-1* mutations are involved in several diseases, such as type 2 Diabetes Mellitus and agenesis of pancreas [17, 39]. The expression of *PDX-1* appears in the early stages of pancreatic cancer and is highly involved in the tumorigenesis [6]. Even though no mutation was reported in the exonic regions of these two genes (*ZIP4* and *PDX-1*) in both human and mouse pancreatic cancer, they play critical roles in pancreatic cancer pathogenesis and progression [4–6]. Since this study only analyzes the exonic DNA, as opposed to the promoters, untranslated regions (UTR) and introns, it is possible that genetic variations (mutations or SNPs) in other regions of these genes may play important roles in gene expression and function in pancreatic cancer.

In an attempt to analyze the genomic sequence of six key genes in two mouse pancreatic cancer cell lines and decode the phenotypic difference of these two mouse pancreatic cancer cells, the coding sequences of the six genes were determined by Sanger's sequencing. A novel mutation in *SMAD4* gene was identified in the two mouse pancreatic cancer cells, which led to a truncated protein product. Functional studies indicate that different TGF- β /SMAD signaling pathways were involved in those two mouse cell lines, which may explain the phenotypic difference between the two cells. Intriguingly, the mutations previously reported in these six genes in human pancreatic cancer were not found in the two mouse pancreatic cancer cells, indicating the genetic difference between the mouse and human pancreatic cancer. The accumulation of *KRAS*, *SMAD4*, *CDKN2A* (*p16*) and *TP53* (*p53*) genetic alterations often lead to pancreatic intraepithelial neoplasia (PanIN) and subsequently progress to an invasive tumor. Within PanIN-1 lesions, 20% had mutations in the oncogene *KRAS*, and 30% had mutations in the tumor suppressor gene *CDKN2A* (*p16*). Those are not only the early genetic events; they can also lead to progressed malignancy with increased frequency during the development of pancreatic cancer. Furthermore, mutations of tumor suppressor genes *SMAD4* and *TP53* (*p53*) were observed in PanIN-3 lesions [40]. It is possible that genetic mutations exist in other oncogenes and tumor suppressor genes in addition to those six key genes that we sequenced in this study, which might contribute to the phenotypic and biologic differences between the two mouse pancreatic cancer cell lines. Further studies are needed to elucidate the molecular pathways that lead to this malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Xia Lin and Xin-Hua Feng for providing the wild type SMAD4 plasmid, antibody, and the 3TP-luciferase reporter vector. This work was supported in part by the National Institutes of Health (NIH) grant R21CA133604, R01CA138701 (M. Li), the Don and Coletta McMillian Foundation (RA Gibbs and MC Gingras), and the Vivian L. Smith Department of Neurosurgery at the University of Texas Health Science Center at Houston, Medical School.

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Fig. (1).

Primer design. Multiple primer sets were designed to cover the coding regions of six genes including *KRAS*, *SMAD4*, *CDKN2A* (*p16*), *TP53* (*p53*), *ZIP4*, and *PDX-1*. The green arrow and line indicates the translation initiation site and translation termination site, respectively; the purple region represents the exon of each gene. The blue arrows indicate the amplicon. A padding region was designed before and after the target region to ensure the high fidelity of the sequencing results. For the larger exons that cannot be covered by a single pair of primers, two or three pairs of primers were used so that the entire coding sequences were completely covered. The universal primer was added to the 5' terminus of each primer for sequencing.

(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

Panc02-KRAS-forward ATGAGTACGACCI
 Panc02-KRAS-reverse ATGAGTACGACCI
 Panc02-H7-KRAS-forward ATGAGTACGACCI
 Panc02-H7-KRAS-reverse ATGAGTACGACCI
 normal C57BL/6 mouse -KRAS-forward ATGAGTACGACCI
 normal C57BL/6 mouse -KRAS-reverse ATGAGTACGACCI
 Reference sequence ATGAGTATGACCI

Panc02-KRAS-forward

Panc02-KRAS-reverse

Panc02-H7-KRAS-forward

Panc02-H7-KRAS-reverse

normal C57BL/6 mouse -KRAS-forward

normal C57BL/6 mouse -KRAS-reverse

Fig. (2).

A synonymous SNP in *KRAS* gene was identified in C57BL/6 normal pancreas and in the two derived pancreatic cancer cell lines. This SNP led to a TAT to TAC coding sequence change without an amino acid sequence change at codon 32.

Panc02-SMAD4-forward
Panc02-SMAD4-reverse
Panc02-H7-SMAD4-forward
Panc02-H7-SMAD4-reverse
normal C57BL/6 mouse -SMAD4-forward
normal C57BL/6 mouse -SMAD4-reverse
Reference sequence

Fig. (3).

A novel non-synonymous mutation of *SMAD4* gene in two mouse pancreatic cancer cell lines. A novel non-synonymous *SMAD4* mutation GAA to TAA at codon 174 was identified in both cells, which led to an amino acid sequence change from Glu to stop codon.

Fig. (4).

No Somatic mutation in *CDKN2A* (*p16*), *TP53* (*p53*), *ZIP4*, and *PDX-1* genes was found in the two mouse pancreatic cancer cells. We did not find any mutation of *CDKN2A* (*p16*), *TP53* (*p53*), *ZIP4* and *PDX-1* in these two mouse pancreatic cancer cell lines.

Fig. (5). SMAD4 protein expression in Panc02, Panc02-H7 and Panc-1 cells. The three cells were examined for the expression of SMAD4 protein. Briefly, cells lysates were extracted and probed with anti-SMAD4 (1:1000), and anti- β -actin (1:10,000) antibodies.

Fig. (6).

Mutant SMAD4 lost its normal function in TGF- β activated SMAD4 dependent signal pathway in mouse pancreatic cancer cells. Panc-1 and Panc02 cells were transfected with 3TP-lux and pcDNA-3.1-mock, pcDNA-3.1-SMAD4-m174 or pcDNA-3.1-SMAD4, respectively. After 24 hours, cells were treated with TGF- β ; The TGF- β induced luciferase activities were then measured. **(A)** Panc-1 cells responded to TGF- β stimulation in all three conditions. **(B)** Panc02 cells did not respond to TGF- β when transfected with mock or mutant SMAD4, but the response to TGF- β was rescued by overexpression of WT SMAD4 in Panc02 cells. * $p < 0.05$.

Table 1

Genetic Variants of Six Key Genes in Pancreatic Cancer

Gene	exon	PCR fragment (Fig. 1)	Alteration	Predict effect	Panc02	Panc02-H7	Reference
KRAS	2	1	GGT to GAT GGT to GTT GGT to CGT	G12D G12V G12R	- - -	- - -	Mu <i>et al.</i> [10]
	2	1	GGC to GAC	G13D	-	-	Brune <i>et al.</i> [24]
	3	3	CAA to CAT	Q61H	-	-	Wistuba <i>et al.</i> [25]
SMAD4	2	2	TAT to TAC	Y32Y	+	+	Current study
	10	10	GGA to TGA	G358end	-	-	Schutte <i>et al.</i> [12]
	11	11	TAC to TAG	Y412end	-	-	Schutte <i>et al.</i> [12]
	12	12	GAT to CAT	H493D	-	-	Schutte <i>et al.</i> [12]
	5	5	GAA to TAA	E174stop	+	+	Current study
CDKN2A	1	1	GGT to GAT	G23D	-	-	Smith-Sorensen <i>et al.</i> [13]
	2	3	CGA to TGA	R80end	-	-	Smith-Sorensen <i>et al.</i> [13]
	2	3	CAC to TAC	H83Y	-	-	Smith-Sorensen <i>et al.</i> [13]
TP53	4	3	CGC to CAC	R175H	-	-	Goh <i>et al.</i> [14]
	6	5	CGG to CAG	R248Q	-	-	Goh <i>et al.</i> [14]
	7	6	CGT to CAT	R273H	-	-	Goh <i>et al.</i> [14]
ZIP4	1	1	TTA to TGA	L48end	-	-	Kury <i>et al.</i> [15]
	1	1	TGT to CGT	C62R	-	-	Kury <i>et al.</i> [15]
	2	2	CCG to CTG	P84L	-	-	Wang <i>et al.</i> [16]
PDX1	1	1	TGC to CGC	C18R	-	-	Macfarlane <i>et al.</i> [17]
	1	1	CAG to CTG	Q59L	-	-	Hani <i>et al.</i> [18]
	2	3	CGC to CAC	R197H	-	-	Macfarlane <i>et al.</i> [17]