Molecular genetics of pancreatic carcinogenesis and their clinical significance

Niki A. Ottenhof

ISBN: 978 90 889 1448 5
Cover design by: Joachim Beekman || Maori sign, Koru (growth)
Printed by: Proefschriftmaken.nl || Uitgeverij BOXPress
Published by: Uitgeverij BOXPress, 's-Hertogenbosch

The research described in this thesis was performed at the department of Pathology of the University Medical Center Utrecht, the Netherlands; and the department of Pathology of the Johns Hopkins University School of Medicine, Baltimore MD, USA

Financial support for the publication of this thesis was kindly profided by the department of pathology of the University Medical Center Utrecht; J.E. Jurriaanse stichting; Menarini Diagnostics; Roche; MRC Holland and Pentax

Molecular genetics of pancreatic carcinogenesis and their clinical significance

Moleculaire genetica van pancreaskanker en de klinische betekenis hiervan

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 6 september 2012 des middags te 12.45 uur

door

Niki Anne Ottenhof
geboren op 18 mei 1986 te *Amsterdam*

Promotoren: Prof. dr. G.J.A. Offerhaus
Prof. dr. C.J.F. van Noorden
Prof. dr. A. Maitra

Chapter 1

Pancreatic cancer, an introductory overview

Chapter 1

Pancreatic cancer, an introductory overview

Annually approximately 44.000 people are diagnosed with pancreatic cancer in the USA. Yearly an estimated 165.000 people die from pancreatic cancer in the developed countries [1]. Because mortality almost equals incidence, pancreatic cancer remains to be the fifth leading cause of cancer-related death throughout the world. Although multiple histological subtypes of pancreatic cancer have been described, pancreatic ductal adenocarcinoma (PDAC) is by far the most commonly observed malignancy in the pancreas. This chapter therefore focuses mainly on PDAC and the terms “pancreatic cancer” and “PDAC” will be used interchangeably.

The aim of this chapter is to give the readers of this thesis a complete introductory overview of this dismal disease. First, the epidemiology, clinical characteristics and clinical management of PDAC are discussed in detail. Second, tumor biology is discussed with special attention to the pre-invasive lesions of PDAC and the genetic alterations found in PDAC. Third, the familial syndromes known to be associated with PDAC are discussed. The last part of this chapter is focused on the current standings of the study of PDAC in experimental animal models.

1. Epidemiology

The incidence of PDAC is approximately 10 per 100.000 people per year in the Western world. Pancreatic cancer is more common in elderly than in younger persons and the peak incidence lies between the sixth and eighth decade of life. Diagnosis with pancreatic cancer before the age of 40 is rare. Its prognosis is poor, largely because most patients present with advanced stages of the disease precluding curative treatment. The vast majority of patients die within one year after diagnosis and the 5-year survival is below 5%. Even after 5 years of survival, recurrence is often observed and true long-term survivors of PDAC are rare. Furthermore, it has been questioned whether the reported 5-year survival rates are correct because large multi-center studies report rates of below 1% [2].

The largest risk factor of PDAC is pancreatitis. Chronic pancreatitis patients have a 5-10-fold increased risk of developing PDAC [3]. Several environmental risk factors for PDAC have been identified as well. Smoking is the most important one, increasing the risk of developing pancreatic cancer by 2-3 fold. Smoking also results in earlier occurrence of PDAC [4, 5]. Other factors that have been reported to increase the risk of PDAC are a diet containing high levels of saturated fat and increased body weight [5, 6]. Diabetes patients appear to have a higher chance of developing PDAC [5]. Although it has often been assumed, alcohol consumption does not seem to be a risk factor unless the alcohol abuse results in pancreatitis [6].

2. Clinical presentation and diagnosis

The early symptoms of PDAC are usually non-specific with abdominal pain, weight loss, anorexia, general malaise, diarrhea and vomiting as the most commonly reported complaints. With progression of the tumor, the pancreatic and bile ducts often become occluded resulting in a decreased secretion of bile and pancreatic juice into the duodenum. Abdominal pain becomes more localized, mainly in the upper middle of the abdomen. The pain is caused primarily by tumor growth into the celiac and superior mesenteric plexus. When the tumor progresses into the retroperitoneal plexus, back pain is often reported. Weight loss is probably caused by decreased food intake in combination with decreased fat uptake by the small intestinal epithelium due to decreased bile secretion. The energy consuming aerobe glycolysis by the tumor cells, known as the Warburg effect, most likely also contributes to the weight loss of PDAC patients.

PDAC can cause malfunctioning of the β-islets in the pancreas leading to diabetes mellitus. At the time of diagnosis, approximately 80% of patients have impaired insulin secretion [7]. Therefore, pancreatic cancer should be included in the differential diagnosis when diabetes type 2 is considered [7]. Remarkably, a dysbalance in plasma glucose levels is often observed in early stages of pancreatic cancer making it a possible marker for PDAC [8].

Physical examination of patients with PDAC often reveals jaundice, a palpable mass in the upper abdomen, hepatomegaly, lymphadenopathy and ascitis. Unfortunately, these signs usually indicate an advanced stage of the tumor. Hematological abnormalities are often non-specific with mild anemia and hyperglycemia often observed. Sometimes, prothrombin time is increased due to malabsorption of vitamin K as a result of decreased fat absorption [9].

Carbohydrate antigen 19-9 (CA19-9) is the most commonly used marker for PDAC. So far, CA19-9 is the only biomarker shown to be useful for therapeutic monitoring and detection of recurrent disease in PDAC [10]. Unfortunately, CA19-9 is only significantly increased in larger tumors making it a disappointing tool for screening [11].

Initial imaging when PDAC is suspected is ultrasonography (US). Although it is difficult to accurately identify magnitude and stage of a tumor, US does provide information on the presence or absence of a pancreatic tumor, bile duct enlargement, liver metastasis and, in combination with Doppler imaging, obstructed blood flow [12]. Although US is a good initial imaging technique, diagnosis and staging is performed by thin-slice contrast-enhanced computed tomography (CT). The technique allows visualization of the tumor in relation to surrounding structures. Therefore, CT is used to evaluate resectability of the tumor as well. Although the negative predictive value for resectablity is high, the positive

predictive value of CT is limited. Approximately 20-50% of patients evaluated to have a resectable tumor reveal to have an unresectable tumor during surgical resection [13]. Positron-emission tomography (PET) scanning is a relatively new technique which shows metabolic activity of cells. It visualizes the tumor on the basis of its higher metabolic rate. It has been shown that, especially in cases where CT scanning fails to identify a discrete mass, PET scanning can be of great value for diagnosing and staging of PDAC [14]. However, CT remains the most commonly used imaging test at present.

3. Staging

PDAC is staged according to the T(umor) N(ode) M(etastasis) classification of the American Joint Committee on Cancer. Currently, the TNM classification of PDAC is as follows: T1, tumor size ≤2.0 cm, limited to the pancreas; T2, tumor size >2.0 cm, limited to the pancreas; T3, tumor extends beyond the pancreas but does not involve the celiac axis or the superior mesenteric artery; T4, tumor is grown into these structures and is therefore unresectable. Lymph node involvement is designated N0 when lymph node metastases are absent and N1 when lymph nodes are involved. When distant metastases are observed the tumor is designated M1. If not, the tumor is designated M0.

For decision making purposes with regard to treatment options, the tumors are divided into stages 1-4 using the TNM classification (Figure 1). Stage I tumors have no lymph node metastases or distant metastases and either a T1 (IA) or T2 (IB) tumor grade. Stage II tumors either have T3N0M0 (IIA) or T1/T2/T3 with lymph node involvement (IIB). Stage III tumors are T4 with or without lymph node involvement and any tumor with distant metastasis is stage IV [15]. Stage III/IV tumors are per definition unresectable.

4. Treatment

Treatment of PDAC differs depending on the resectability of the tumor. Because of the frequent occurrence of advanced stage of the tumor at diagnosis, only 15-20% of tumors are potentially resectable [16, 17]. Dependent on the localization of the tumor, patients undergo either classical pancreaticoduodenectomy, distal pancreatectomy or total pancreatectomy. Adjuvant therapy after resection consists of gemcitabine, which has been the treatment of choice since 1997 when it was shown to improve both disease-free and overall survival [18, 19]. This study showed that median survival after treatment with gemcitabine was lengthened with 1.2 months compared to survival after treatment with fluorouracil, which had been the treatment of choice until then. Also, the survival rate after 12 months was 18%

for gemcitabine and 2% for fluorouracil. Efforts to improve gemcitabine treatment by additional therapeutic agents showed disappointing results [20, 21]. Only addition of erlotinib showed a slight improvement in overall survival [22]. A recently published study confirmed the earlier observed limited beneficial effect of the addition of erlotinib, but the improvement did not justify a phase III trail [23]. Reports comparing single agent gemcitabine therapy to chemoradiation therapy have been inconclusive. Chemoradiation therapy has been implicated in the USA since the Gastro-Intestinal Tumor Study Group trial showed longer overall survival in the group treated with chemoradiation therapy [24, 25]. In Europe, however, a similar study failed to find a significant survival advantage for the patients receiving additional radiotherapy. Therefore, chemoradiation therapy is not the standard in Europe [26]. Although more recent reports suggest a significant advantage of additional radiotherapy, it is still controversial and more research is needed before radiation therapy is included as standard first-line treatment in Europe [25].

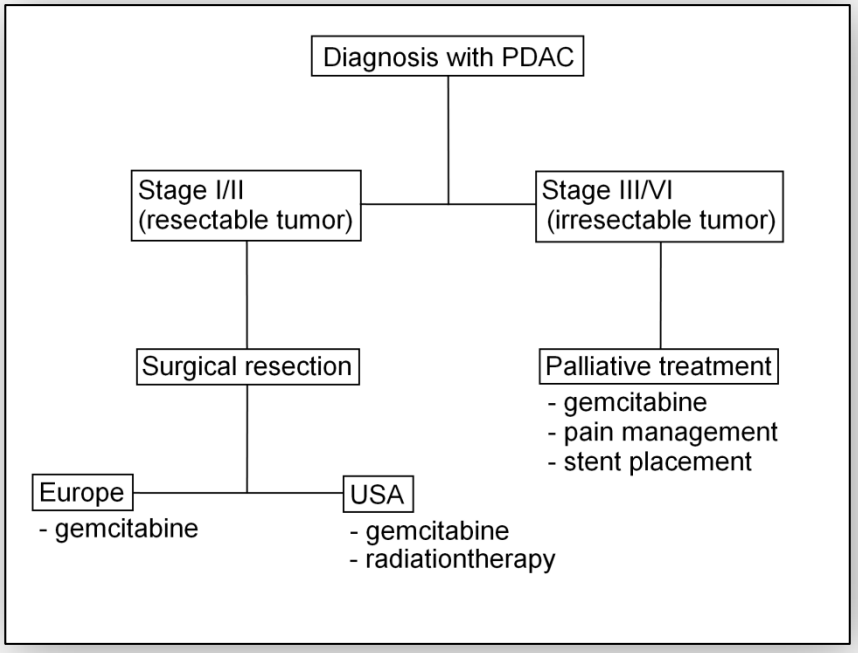


Figure 1 Management of PDAC

As mentioned above, approximately 80% of patients present with locally or systemically advanced disease making resection redundant. For these patients, palliative treatment options remain. Single agent gemcitabine is currently recommended as standard first-line chemotherapy for patients with advanced

disease [18]. However, a recent study showed a significant increase in survival upon adding radiotherapy to gemcitabine treatment in patients with locally advanced cancer [27]. Apart from gemcitabine, no other treatment options are standard for advanced stage pancreatic cancer. It has been suggested that neoadjuvant treatment in combination with chemotherapy, radiation therapy or chemoradiation therapy can downstage borderline resectable tumors. Several studies have shown promising results for the treatment of borderline resectable tumors with chemoradiation, enabling resection. This type of treatment results in similar survival rates as those obtained with resectable tumors [28-31].

Pain control is a very important part of palliative treatment of patients with PDAC. Intervention by neurolysis with chemical agents can significantly decrease pain [32]. Obstructive jaundice, which occurs in as many as 80% of patients with PDAC, can generally be relieved by endoscopic stent placement [33]. Although palliative chemotherapy was shown to prolong survival, it does not improve quality of life, whereas both pain control and stent placement significantly improve quality of life [34].

5. Tumor biology

5.1 Pre-invasive lesions

PDAC, like all cancers, is essentially a genetic disease in which alterations in oncogenes, tumor-suppressor genes and genome maintenance genes eventually lead to tumor development. Over the last two decades, large steps have been taken in the understanding of the pathogenesis of PDAC. It is now well accepted that PDAC develops in a step-wise manner in which non-malignant pre-invasive lesions progress to an invasively growing tumor. These pre-invasive lesions of PDAC are called pancreatic intraepithelial neoplasia (PanIN) lesions [35, 36]. The lesions have microscopic dimensions and are found in smaller pancreatic ducts. They are classified into different stages on the basis of cellular and nuclear atypia and architectural changes. PanIN-1 lesions show little cytonuclear atypia and have retained their cellular polarity. These type 1 lesions are subdivided into PanIN-1A and PanIN-1B lesions based on whether the cells are organized in a squamous-like fashion (1A) or show micropapillary architecture (1B). PanIN-2 lesions show evident cytonuclear atypia and infrequent mitoses. PanIN-3 lesions, also referred to as carcinoma in situ, show all the hallmarks of cancer except the invasive growth.

5.2 Genetic alterations

Progression from normal pancreatic tissue to invasively growing tumor is driven by genetic alterations. Telomere shortening is one of the first events in the

progression to a pre-invasive lesion and eventually to PDAC. It is observed in virtually all pre-invasive lesions [37] and results in chromosomal instability that allows accumulation of genetic alterations. Telomeres are repeat sequences at the end of linear chromosomes that prevent fusion between the ends of chromosomes. Pathologically short telomeres can result in ring chromosomes and dicentric chromosomes that form so-called anaphase bridges during mitosis. Breakage of these anaphase bridges generates highly recombinogenic free DNA ends, which in turn can result in chromosomal rearrangements. These so-called anaphase bridge-breakage-fusion cycles are repeated, thereby creating the genetic instability that forms the basis of tumor development.

The oncogene *KRAS2* is one of the major players in the development of PDAC. *KRAS2* is found to be mutated in over 90% of PDAC cases. The gene is also found to be mutated in early pre-invasive lesions. *KRAS2* mutations are observed in 10-30% of PanIN-1 lesions, 45% of PanIN-2 lesions and 85% of PanIN-3 lesions [38]. Mouse model experiments have identified *KRAS2* mutations to be the initiating event in pancreatic carcinogenesis; this will be discussed in further detail below.
The most commonly affected tumor suppressor gene in PDAC is *CDKN2A*, which encodes for protein p16. Inactivation of *CDKN2A* is found in >95% of PDAC cases [39]. When assessing p16 expression by immunohistochemistry, absence of nuclear staining indicates inactivation of the protein and is observed in, respectively, 30%, 50%, and 70% of PanIN-1, PanIN-2 and PanIN-3 lesions [40]. Another tumor suppressor gene, *TP53*, is found to be inactivated in 50-75% of the PDACs [41]. *TP53* is the most commonly affected gene in human cancer. The gene encodes p53, a protein involved in cell-cycle arrest and apoptosis upon cytotoxic stress. *TP53* mutations are observed mainly in PanIN-3 lesions in transition to invasive growth warranting p53 defects as a late event in PDAC development [42]. The cell-cycle arrest mediated by p53 is realized by activation of another protein, p21. P21 inhibits the activity of a cell proliferation-stimulating complex containing the protein cyclin D. Activity of p21 is lost in 40-60% of PDACs [43, 44].
Mutations in *DPC4*, another tumor suppressor gene, are found in approximately 55% of the PDACs and like *TP53* mutations, *DPC4* mutations occur in a late stage in tumor development and are found only in PanIN-3 lesions [45]. Smad4, the protein product of *DPC4* is part of the transforming growth factor β (TGF-β) pathway. TGF-β signaling is one of the most potent cell proliferation inhibitors but has many other cellular functions including differentiation, apoptosis and angiogenesis [46]. In pancreatic cancer, *DPC4* is of specific interest because persistent *DPC4* expression is related to a better prognosis rendering the Smad4 of high diagnostic value in PDAC [47-49]. Moreover, immunohistochemical staining of

Smad4 accurately mirrors its gene expression making experimental investigation of DPC4 expression relatively easy [48].

Another set of genes regularly found mutated in sporadic PDAC is that of the mismatch repair genes. These genes encode for DNA repair proteins. When these genes are affected, genomic instability may occur and genetic alterations can accumulate. Mismatch repair genes are mutated in 4-10% of PDAC cases and defects in these genes results in microsatellite instability (MSI) [50, 51]. Microsatellites are repeat sequences of DNA and mutations in the mismatch repair genes lead to pathologically short or long microsatellites. MSI results in a typical 'medullary' appearance of the tumors which includes syncytia, pushing borders and lymphocyte infiltration. Microsatellite-instable tumors have a slightly better prognosis than their microsatellite-stable counterparts, possibly because the genomic instability results in a higher probability for fatal mutations to occur in these tumors [51]. Another DNA repair gene involved in pancreatic cancer is *BRCA2*. This gene is found to be mutated in 7-10% of the sporadic PDACs and in 10% of familial PDACs [52]. *BRCA2* mutations, best known for their involvement in hereditary breast and ovarian cancer, are involved in the cancer-susceptibility disorder Fanconi anemia. Mutations in *FANCC* and *FANCG* cause Fanconi anemia but are also involved in PDAC [53]. Because *BRCA2*, *FANCC* and *FANCG* are involved in interstrand crosslinking repair, tumors harboring defects in these genes have been reported to better respond to treatment with mitomycin C, cisplatin, chlorambucil, and melphalan, all chemotherapeutics that induce this type of DNA damage [54].

Another genomic feature that has been related to PDAC is re-activation of embryonic signaling pathways. The Notch pathway, usually inactivated in the normal pancreas, was reported to be active in a large proportion of PDACs [55, 56], whereas Notch pathway activation increased the number of PanIN lesions in genetically-engineered mice exhibiting mutant Kras expression [57]. Another embryonic signaling pathway often found to be reactivated in PDAC is the Hedgehog pathway. Surprisingly, epithelial cells in the tumor do not respond to Hedgehog signaling and most recent studies point towards a role for Hedgehog signaling in the maintenance of the extensive stromal compartment of the PDACs. Inhibition of Hedgehog signaling in xenograft tumors in mice reduces tumor growth, largely because of a smaller stromal compartment [58]. Moreover, disruption of Hedgehog signaling in a transgenic mouse model increased the response to chemotherapy through better accessibility of the cancer by inhibition of development of tumoral stroma [59].

6. Inheritance

Approximately 10% of the PDAC patients have a family history of the disease. A first degree relative with PDAC increases the risk of developing this disease by 2-fold and this risk increases with number of family members affected. However, often a genetic basis for this familial trait can not be identified [60]. In some cases, PDACs arise in relation to a familial cancer syndrome caused by germline mutations. At least 5 cancer syndromes have been described that increase the risk to develop PDAC. One of these cancer syndromes is hereditary pancreatitis. The syndrome is caused by mutations in either the *PRSS1* gene or the *SPINK1* gene. Patients suffering from hereditary pancreatitis present with recurrent episodes of pancreatitis from an early age on and have a life time risk to develop PDAC of 25-50% at the age of 60 [61, 62].

Another syndrome related to PDAC is the Peutz-Jeghers syndrome. This syndrome is caused by mutations in the tumor suppressor gene *LKB1* and results in multiple polyps throughout the gastro-intestinal tract. Besides a high life time risk to develop colorectal cancer, these patients have a >100-fold increased chance to develop PDAC [63, 64].

The third cancer syndrome increasing the risk of developing PDAC is the familial atypical multiple melanoma and mole (FAMMM) syndrome. This syndrome is caused by mutations in the *CDKN2A* gene, already discussed above, and results in multiple unusual nevi and melanomas. Patients suffering from the FAMMM syndrome have a 20-34 increased risk to develop PDAC [65].

The Lynch syndrome, yet another syndrome involved in hereditary PDAC, is caused by mutations in mismatch repair genes. Patients suffering from the Lynch syndrome predominantly present with multiple polyps in the gastrointestinal tract which have a tendency to progress into malignant growth. However, it has been demonstrated that the Lynch syndrome increased the chance to develop PDAC slightly, although it is still unclear what the exact role of the syndrome is in PDAC [50, 66, 67].

The last syndrome known at present to be related to hereditary PDAC is familial breast and ovarian cancer. The syndrome is cause by alterations in the *BRCA1/BRCA2* genes. Although germline mutations in *BRCA1* do not seem to be related with a higher risk to develop PDAC, *BRCA2* mutations increase this risk by 3-10 fold [68]. *BRCA2* mutations are responsible for ~10% of all familial PDAC cases [69].

Besides the five cancer syndromes known to increase the risk of PDAC, a genetic basis for the familial pattern of PDAC development has not been identified. Further research on familial PDAC is needed to completely understand the hereditary component of PDAC.

7. Mouse models

Mouse models are an important tool in cancer research. There are three major mouse models available at present: the xenograft implanted mouse model, the carcinogen-induced mouse model and the genetically-engineered mouse model. All these models have their own advantages and disadvantages but the genetically engineered mouse model recapitulates the development of the human disease best. The use of genetically-engineered mice in pancreatic cancer research has boomed since 2003 when Hingorani and colleagues [70] created a mouse model for PDAC by conditional knock-in of mutant $Kras^{G12D}$. Mutant Kras expression was limited to the pancreas and embryonic foregut by targeting only Pdx1-positive cells. Pdx1 is a transcription factor necessary for pancreatic development and is selectively expressed by epithelial cells that give rise to pancreatic formation. The mice expressing mutant Kras developed PanIN lesions of various grades with occasional progression to invasive carcinoma [70]. When inducing additional mutations in the mice in $p16^{INK4a}$ /$p19^{Arf}$ [71], p53 [72], or $p16^{INK4a}$/p53, this malignant progression was expedited with higher penetrance, earlier onset, progression to distant metastasis and reduced survival [71, 72].
More recently, the TGFβ signaling pathway was targeted in mouse models. Conditional gene knock out of *SMAD4* or *TGFβRII* alone did not result in pre-invasive lesions or malignant progression but when combining these knock outs with $Kras^{G12D}$ expression, a tremendous increase in carcinogenesis occurred with 100% penetration [73, 74]. These mouse model experiments have provided major insights in PDAC development. Most importantly, the role of Kras in initiating PDAC development was elucidated. Although many other genetic alterations were proven to expedite tumor development when combined with mutant Kras expression, only $Kras^{G12D}$ expression initiated PanIN lesions that resembled human pre-invasive lesions. This finding explains the high percentage of Kras mutations found in pre-invasive lesions in humans. Furthermore, the mouse models have enabled the search for the cell of origin in PDAC. Limiting mutant expression to certain cell types enabled the investigation of cells that are at the basis of PDAC development. For instance, limiting $Kras^{G12D}$ expression to the elastase-producing (centro)acinar cells resulted in mouse-PanIN formation, albeit only with concurrent induction of chronic pancreatitis [75]. The greatest advantage of these models is probably the possibility to study new therapeutic strategies in a setting that closely recapitulates human disease progression.

8. Conclusion and future perspectives

Intensive research over the last two decades has resulted in a solid understanding of carcinogenesis in the pancreas. Unfortunately, this understanding of disease development has not lead to major advanced in diagnostics or treatment and the disease is still virtually always lethal. The main problem with PDAC is the high rate

of unresectable tumors when diagnosed and the high percentage of tumor recurrence despite complete resection. The emphasis of future research should be on the development of screening methods for PDAC and the identification of tumor markers for early stages of the disease. Furthermore, the development of new (targeted) treatment strategies that increase survival time and decrease recurrence rates is of vital importance.

Reference list

1. Siegel R, Naishadham D and Jemal A, *Cancer statistics, 2012.* CA Cancer J Clin, 2012. 62(1): p. 10-29.
2. Gudjonsson B, *Pancreatic cancer: survival, errors and evidence.* Eur J Gastroenterol Hepatol, 2009. 21(12): p. 1379-82.
3. Raimondi S, Lowenfels AB, Morselli-Labate AM, et al., *Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection.* Best Pract Res Clin Gastroenterol, 2010. 24(3): p. 349-58.
4. Anderson LN, Cotterchio M, and Gallinger S, *Lifestyle, dietary, and medical history factors associated with pancreatic cancer risk in Ontario, Canada.* Cancer Causes Control, 2009. 20(6): p. 825-34.
5. Stevens RJ, Roddam AW, Spencer EA, et al., *Factors associated with incident and fatal pancreatic cancer in a cohort of middle-aged women.* Int J Cancer, 2009. 124(10): p. 2400-5.
6. Zhang, J. Dhakal IB, Gross MD, et al., *Physical activity, diet, and pancreatic cancer: a population-based, case-control study in Minnesota.* Nutr Cancer, 2009. 61(4): p. 457-65.
7. Permert J. Adrian TE, Jacobsson P, et al., *Is profound peripheral insulin resistance in patients with pancreatic cancer caused by a tumor-associated factor?* Am J Surg, 1993. 165(1): p. 61-6; discussion 66-7.
8. Chari ST, Leibson CL, Rabe KG, et al., *Pancreatic cancer-associated diabetes mellitus: prevalence and temporal association with diagnosis of cancer.* Gastroenterology, 2008. 134(1): p. 95-101.
9. Maitra A and Hruban RH, *Pancreatic cancer.* Annu Rev Pathol, 2008. 3: p. 157-88.
10. Harsha HC, Kandasamy K, Ranganathan P, et al., *A compendium of potential biomarkers of*
pancreatic cancer. PLoS Med, 2009. 6(4): p. e1000046.
11. Barton CM, Staddon SL, Hughes CM, et al., *Predictive and prognostic value of CA 19-9 in resected pancreatic adenocarcinoma.* J Gastrointest Surg, 2009. 13(11): p. 2050-8.
12. Clarke DL, Thomson SR, Madiba TE, et al., *Preoperative imaging of pancreatic cancer: a management-oriented approach.* J Am Coll Surg, 2003. 196(1): p. 119-29.
13. Potter MW, Shah SA, McEnaney P, et al., *A critical appraisal of laparoscopic staging in hepatobiliary and pancreatic malignancy.* Surg Oncol, 2000. 9(3): p. 103-10.
14. Serrano OK, Chaudhry MA and Leach SD, *The role of PET scanning in pancreatic cancer.* Adv Surg, 2010. 44: p. 313-25.
15. Edge S, Byrd D, and Compton C, *Exocrine and endocrine pancreas*, in *AJCC cancer staging manual*. 2010, Springer: New York. p. 241-9.
16. Geer RJ and Brennan MF, *Prognostic indicators for survival after resection of pancreatic adenocarcinoma.* Am J Surg, 1993. 165(1): p. 68-72; discussion 72-3.
17. Smeenk HG, Tran TC, Erdmann J, et al., *Survival after surgical management of pancreatic adenocarcinoma: does curative and radical surgery truly exist?* Langenbecks Arch Surg, 2005. 390(2): p. 94-103.
18. Burris HA, 3rd, Moore MJ, Andersen J, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* J Clin Oncol, 1997. 15(6): p. 2403-13.
19. Oettle H, Post S, Neuhaus P, et al., *Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial.* Jama, 2007. 297(3): p. 267-77.

20. Kullmann F, Hollerbach S, Dollinger MM, et al., *Cetuximab plus gemcitabine/oxaliplatin (GEMOXCET) in first-line metastatic pancreatic cancer: a multicentre phase II study.* Br J Cancer, 2009. 100(7): p. 1032-6.
21. Kindler HL, Niedzwiecki D, Hollis D, et al., *Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303).* J Clin Oncol, 2010. 28(22): p. 3617-22.
22. Moore MJ, Goldstein D, Hamm J, et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* J Clin Oncol, 2007. 25(15): p. 1960-6.
23. Feliu J, Borrega P, Leon A, et al., *Phase II study of a fixed dose-rate infusion of gemcitabine associated with erlotinib in advanced pancreatic cancer.* Cancer Chemother Pharmacol, 2010.
24. Kalser MH and Ellenberg SS, *Pancreatic cancer. Adjuvant combined radiation and chemotherapy following curative resection.* Arch Surg, 1985. 120(8): p. 899-903.
25. Hsu CC, Herman JM, Corsini MM, et al., *Adjuvant chemoradiation for pancreatic adenocarcinoma: the Johns Hopkins Hospital-Mayo Clinic collaborative study.* Ann Surg Oncol, 2010. 17(4): p. 981-90.
26. Klinkenbijl JH, Jeekel J, Sahmoud T, et al., *Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region: phase III trial of the EORTC gastrointestinal tract cancer cooperative group.* Ann Surg, 1999. 230(6): p. 776-82; discussion 782-4.
27. Shibuya K, Oya N, Fujii T, et al., *Phase II study of radiation therapy combined with weekly low-dose gemcitabine for locally advanced, unresectable pancreatic cancer.* Am J Clin Oncol, 2010.
28. Jessup JM, Steele G, Jr., Mayer RJ, et al., *Neoadjuvant therapy for unresectable pancreatic adenocarcinoma.* Arch Surg, 1993. 128(5): p. 559-64.
29. Pilepich MV and Miller HH, *Preoperative irradiation in carcinoma of the pancreas.* Cancer, 1980. 46(9): p. 1945-9.
30. Brunner TB, Grabenbauer GG, Kastl S, et al., *Preoperative chemoradiation in locally advanced pancreatic carcinoma: A phase II study.* Onkologie, 2000. 23(5): p. 436-442.
31. Brown KM, Siripurapu V, Davidson M, et al., *Chemoradiation followed by chemotherapy before resection for borderline pancreatic adenocarcinoma.* Am J Surg, 2008. 195(3): p. 318-21.
32. Lillemoe KD, Cameron JL, Kaufman HS, et al., *Chemical splanchnicectomy in patients with unresectable pancreatic cancer. A prospective randomized trial.* Ann Surg, 1993. 217(5): p. 447-55; discussion 456-7.
33. Andersen JR, Sorensen SM, Kruse A, et al., *Randomised trial of endoscopic endoprosthesis versus operative bypass in malignant obstructive jaundice.* Gut, 1989. 30(8): p. 1132-5.
34. Crippa S, Dominguez I, Rodriguez JR, et al., *Quality of life in pancreatic cancer: analysis by stage and treatment.* J Gastrointest Surg, 2008. 12(5): p. 783-93; discussion 793-4.
35. Hruban RH, Adsay NV, Albores-Saavedra J, et al., *Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions.* Am J Surg Pathol, 2001. 25(5): p. 579-86.
36. Hruban RH, Takaori K, Klimstra DS, et al., *An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms.* Am J Surg Pathol, 2004. 28(8): p. 977-87.

37. van Heek NT, Meeker AK, Kern SE, et al., *Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia.* Am J Pathol, 2002. 161(5): p. 1541-7.
38. Lohr M, Kloppel G, Maisonneuve P, et al., *Frequency of K-ras mutations in pancreatic intraductal neoplasias associated with pancreatic ductal adenocarcinoma and chronic pancreatitis: a meta-analysis.* Neoplasia, 2005. 7(1): p. 17-23.
39. Schutte M, Hruban RH, Geradts J, et al., *Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas.* Cancer Res, 1997. 57(15): p. 3126-30.
40. Wilentz, R.E., et al., *Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression.* Cancer Res, 1998. 58(20): p. 4740-4.
41. Barton, C.M., et al., *Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer.* Br J Cancer, 1991. 64(6): p. 1076-82.
42. Koorstra, J.B., et al., *Widespread activation of the DNA damage response in human pancreatic intraepithelial neoplasia.* Mod Pathol, 2009. 22(11): p. 1439-45.
43. Harada, N., et al., *Nuclear accumulation of p53 correlates significantly with clinical features and inversely with the expression of the cyclin-dependent kinase inhibitor p21(WAF1/CIP1) in pancreatic cancer.* Br J Cancer, 1997. 76(3): p. 299-305.
44. Ahrendt, S.A., et al., *p21WAF1 expression is associated with improved survival after adjuvant chemoradiation for pancreatic cancer.* Surgery, 2000. 128(4): p. 520-30.
45. Wilentz RE, Geradts J, Maynard R, et al., *Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression.* Cancer Res, 2000. 60(7): p. 2002-6.
46. Miyazono K, ten Dijke P and Heldin CH, *TGF-beta signaling by Smad proteins.* Adv Immunol, 2000. 75: p. 115-57.
47. Tascilar M, Offerhaus GJ, Altink R, et al., *Immunohistochemical labeling for the Dpc4 gene product is a specific marker for adenocarcinoma in biopsy specimens of the pancreas and bile duct.* Am J Clin Pathol, 2001. 116(6): p. 831-7.
48. Tascilar M, Skinner HG, Rosty C, et al., *The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma.* Clin Cancer Res, 2001. 7(12): p. 4115-21.
49. Iacobuzio-Donahue CA, Fu B, Yachida S, et al., *DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer.* J Clin Oncol, 2009. 27(11): p. 1806-13.
50. Goggins M, Offerhaus GJ, Hilgers W, et al., *Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+.* Am J Pathol, 1998. 152(6): p. 1501-7.
51. Nakata B, Wang YQ, Yashiro M, et al., *Prognostic value of microsatellite instability in resectable pancreatic cancer.* Clin Cancer Res, 2002. 8(8): p. 2536-40.
52. Goggins M, Schutte M, Lu J, et al., *Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas.* Cancer Res, 1996. 56(23): p. 5360-4.
53. van der Heijden MS, Yeo CJ, Hruban RH, et al., *Fanconi anemia gene mutations in young-onset pancreatic cancer.* Cancer Res, 2003. 63(10): p. 2585-8.
54. van der Heijden MS, Brody JR, Dezentje DA, et al., *In vivo therapeutic responses contingent on Fanconi anemia/BRCA2 status of the tumor.* Clin Cancer Res, 2005. 11(20): p. 7508-15.
55. Miyamoto Y, Maitra A, Ghosh B, et al., *Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis.* Cancer Cell, 2003. 3(6): p. 565-76.

56. Mullendore ME, Koorstra JB, Li YM, et al., *Ligand-dependent Notch signaling is involved in tumor initiation and tumor maintenance in pancreatic cancer.* Clin Cancer Res, 2009. 15(7): p. 2291-301.
57. De La OJ, Emerson LL, Goodman JL, et al., *Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia.* Proc Natl Acad Sci U S A, 2008. 105(48): p. 18907-12.
58. Yauch RL, Gould SE, Scales SJ, et al., *A paracrine requirement for hedgehog signalling in cancer.* Nature, 2008. 455(7211): p. 406-10.
59. Feldmann G, Habbe N, Dhara S, et al., *Hedgehog inhibition prolongs survival in a genetically engineered mouse model of pancreatic cancer.* Gut, 2008. 57(10): p. 1420-30.
60. Shirts BH, Burt RW, Mulvihill SJ, et al., *A population-based description of familial clustering of pancreatic cancer.* Clin Gastroenterol Hepatol, 2010. 8(9): p. 812-6.
61. Lowenfels AB, Maisonneuve P, DiMagno EP, et al., *Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group.* J Natl Cancer Inst, 1997. 89(6): p. 442-6.
62. Howes N, Lerch MM, Greenhalf W, et al., *Clinical and genetic characteristics of hereditary pancreatitis in Europe.* Clin Gastroenterol Hepatol, 2004. 2(3): p. 252-61.
63. Giardiello FM, Brensinger JD, Tersmette AC, et al., *Very high risk of cancer in familial Peutz-Jeghers syndrome.* Gastroenterology, 2000. 119(6): p. 1447-53.
64. Giardiello FM, Welsh SB, Hamilton SR, et al., *Increased risk of cancer in the Peutz-Jeghers syndrome.* N Engl J Med, 1987. 316(24): p. 1511-4.
65. Bartsch DK, Sina-Frey M, Lang S, et al., *CDKN2A germline mutations in familial pancreatic cancer.* Ann Surg, 2002. 236(6): p. 730-7.
66. Wilentz RE, Goggins M, Redston M, et al., *Genetic, immunohistochemical, and clinical features of medullary carcinoma of the pancreas: A newly described and characterized entity.* Am J Pathol, 2000. 156(5): p. 1641-51.
67. Kastrinos F, Mukherjee B, Tayob N, et al., *Risk of pancreatic cancer in families with Lynch syndrome.* Jama, 2009. 302(16): p. 1790-5.
68. Hruban RH, Petersen GM, Goggins M, et al., *Familial pancreatic cancer.* Ann Oncol, 1999. 10 Suppl 4: p. 69-73.
69. van der Heijden MS, Brody JR, Gallmeier E, et al., *Functional defects in the fanconi anemia pathway in pancreatic cancer cells.* Am J Pathol, 2004. 165(2): p. 651-7.
70. Hingorani SR, Petricoin EF, Maitra A, et al., *Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse.* Cancer Cell, 2003. 4(6): p. 437-50.
71. Aguirre AJ, Bardeesy N, Sinha M, et al. *Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma.* Genes Dev, 2003. 17(24): p. 3112-26.
72. Hingorani SR, Wang L, Multani AS, et al., *Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice.* Cancer Cell, 2005. 7(5): p. 469-83.
73. Bardeesy N, Cheng KH, Berger JH, et al., *Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer.* Genes Dev, 2006. 20(22): p. 3130-46.
74. Ijichi H, Chytil A, Gorska AE, et al., *Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression.* Genes Dev, 2006. 20(22): p. 3147-60.
75. Guerra C, Schuhmacher AJ, Canamero M, et al., *Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice.* Cancer Cell, 2007. 11(3): p. 291-302.

Chapter 2

Outline of the thesis

Outline of the thesis

Although pancreatic cancer research has been fruitful over the past decades, the disease remains extremely devastating. Progression from a normal pancreatic cell to a malignant tumor is better understood now than 20 years ago, but these insights have not yet been translated into advances in prognosis or outcome of the disease. Like all types of cancer, pancreatic ductal adenocarcinoma (PDAC), the most common pancreatic malignancy, is a disease of the genes and the genetic alterations that are involved in the development of PDAC have been under investigation for many years. It is now well established that non-malignant pre-invasive lesions precede malignant tumor development and that the accumulation of genetic changes drives this malignant transformation. Current goals in PDAC research have to be the development of specific molecular diagnostic markers for early detection of PDAC since most patient present with incurable tumors. Moreover, the investigation of prognostically relevant alterations may increase our understanding of the malignant behavior of the tumor and may result in new treatment targets. Further investigations based on the insights provided by genetic and proteomic research seem to be the road to develop new treatment strategies and efforts should be put into understanding the effects that various genetic alterations have on tumor development and tumor progression.

In order to provide a comprehensive overview of the current insights in pancreatic carcinogenesis, the review articles in **Chapter 3** and **Chapter 4** are included in the thesis. Chapter 3 focuses mainly on the step-wise progression from a normal pancreatic cell to a malignant cell. It highlights the genetic changes that take place in the different stages of the pre-invasive lesions of PDAC, called pancreatic intraepithelial neoplasia. Special attention is paid to the mouse models currently used for pancreatic cancer research which faithfully recapitulate human tumor development. In chapter 4, the genetic alterations in PDAC are presented, in the context of their role in signaling pathways. Moreover, the chapter describes recent advances in the development of targeted treatment of PDAC.

Over the last decades, various clinical and pathological features of the prognosis of PDAC have been reported. These features are usually evaluated separately without the use of multivariate analysis. In **Chapter 5,** a cohort of 78 PDAC patients with varying survival time is described. A wide range of clinical characteristics of these patients were evaluated. Moreover, immunohistochemical analysis of a series of proteins, that have been reported to affect survival, was performed. Multivariate analysis was conducted in order to study which clinicopathological features have independent prognostic significance.

Chapter 6 also focuses on the genetic changes in PDAC in relation to prognosis and survival. The databases of 3 different cancer treatment centers were searched for very long-term survivors of PDAC and a unique cohort of 24 >10-year survivors was formed. In an attempt to address the question why these patients survived against all odds, multiple clinical and pathological features were evaluated in this very long-term survival cohort and were compared with the data from the literature and a short-term survival control group. The cohort was screened for alterations in

genes most commonly affected in PDAC using RT-PCR, sequencing and immunohistochemistry.
This very long-term survivor cohort is further studied in **Chapter 7.** This chapter describes changes in protein expression. Five of the very long-term survivors were stage-matched with a short-term survival control group and quantitative proteomics was applied in search for differentially expressed proteins that affect survival. From the proteins that were found to be differentially expressed in the very long-term survival cohort, galectin-1 was selected for further testing to evaluate whether expression of this protein predicts survival in PDAC.
As discussed earlier, various signaling pathways show altered activity in PDAC. Among these are several embryonic pathways and in **Chapter 8** one of these embryonic pathways, the Notch pathway, is described in more detail. Notch signaling has been shown to be activated in several malignancies among which PDAC. However, it is not completely understood how Notch signaling affects tumor development. In order to delineate the role of Notch signaling in PDAC development and progression, a receptor-specific Notch-1 antibody was used to block Notch signaling in PDAC cell lines. After treatment with the antibody, different functional assays were conducted including a cell viability assay and an anchorage-independent growth assay to investigate how Notch signaling affects these features in cancer cells. Moreover, the potential of Notch inhibitors in treating PDAC was evaluated.
In 1997, gemcitabine was implicated as first line treatment after resection of PDAC. The addition of other chemotherapeutics, monoclonal antibodies or radiation to gemcitabine treatment did not result in any meaningful improvement of survival of PDAC patients. In **Chapter 9**, a new treatment option in PDAC is discussed. The efficacy and pharmacodynamic effects of MK-1775, a potent Wee1 inhibitor, in both monotherapy and in combination with gemcitabine was evaluated using a panel of p53-deficient and p53 wild-type human PDAC xenografts.

Genetic alterations are the driving force behind the development of cancer and therefore a germline mutation in a relevant gene could increase the chance to develop a tumor. As described in the general introduction, a number of hereditary cancer syndromes have been found to increase the lifetime chance to develop PDAC. However, the occurrence of PDAC in a patient suffering from a syndrome not previously associated with PDAC raised the question whether the germline mutation caused PDAC. In **Chapter 10,** a remarkable case is described of a patient who suffered from hereditary gastric cancer caused by a germline mutation in the *CDH1* gene and was subsequently diagnosed to suffer from PDAC. The *CDH1* gene encodes for the protein E-cadherin, which is involved in cell-cell adhesion. Downregulation of *CDH1* has been described in PDAC and our aim was to evaluate whether the germline mutation in this gene induced PDAC. To this end, expression of the E-cadherin protein was evaluated using immunohistochemistry. Moreover, alterations in the *CDH1* gene were evaluated using Sanger sequencing.
Finally, **Chapter 11** describes another type of pancreatic cancer, namely the acinar cell carcinoma (ACC). ACCs are responsible for approximately 1-2% of all exocrine

pancreatic malignancies and because of the relative rarity of this malignancy not much is known about its tumorigenesis. The occurrence of an ACC in a Peutz-Jehgers syndrome patient raised the question whether *LKB1*, the gene responsible for the Peutz-Jehgers syndrome, affects ACC development. A panel of 5 ACCs was selected and screened for mutations in *LKB1* or alterations in *LKB1* expression. Moreover, several genes involved in the development of PDAC were evaluated in the ACC panel to better understand ACC carcinogenesis.

The thesis ends with a summary with concluding remarks in both English and Dutch in **chapters 12** and **13,** respectively.

Chapter 3

Pancreatic intraepithelial neoplasia and pancreatic tumorigenesis: of mice and men

Niki A. Ottenhof, Anya N. A. Milne, Folkert H. M. Morsink, Paul Drillenburg, Fiebo J. W. ten Kate, Anirban Maitra, G. Johan A. Offerhaus

Archives of Pathology & Laboratory Medicine 2009; 133: 375-381.

Chapter 3

Abstract

Context.—Pancreatic cancer has a poor prognosis with a 5-year survival of less than 5%. Early detection is at present the only way to improve this outlook. This review focuses on the recent advances in our understanding of pancreatic carcinogenesis, the scientific evidence for a multistaged tumor progression, and the role genetically engineered mouse models can play in recapitulating the natural course and biology of human disease.

Objectives.—To illustrate the stepwise tumor progression of pancreatic cancer and genetic alterations within the different stages of progression and to review the findings made with genetically engineered mouse models concerning pancreatic carcinogenesis.

Data Sources.—A review of recent literature on pancreatic tumorigenesis and genetically engineered mouse models.

Conclusions.—Pancreatic cancer develops through stepwise tumor progression in which preinvasive stages, called pancreatic intraepithelial neoplasia, precede invasive pancreatic cancer. Genetic alterations in oncogenes and tumor suppressor genes underlying pancreatic cancer are also found in pancreatic intraepithelial neoplasia. These mutations accumulate during progression through the consecutive stages of pancreatic intraepithelial neoplasia lesions. Also in genetically engineered mouse models of pancreatic ductal adenocarcinoma, tumorigenesis occurs through stepwise progression via consecutive mouse pancreatic intraepithelial neoplasia, and these models provide important tools for clinical applications. Nevertheless differences between mice and men still remain.

Introduction

Pancreatic cancer has a poor prognosis. Incidence rates equal mortality rates and even though pancreatic cancer is not one of the most common cancers, it ranks fourth as cause of cancer-related mortality [1]. Despite advances in surgical and medical treatment, the 5-year survival rate has remained less than 5% [2].

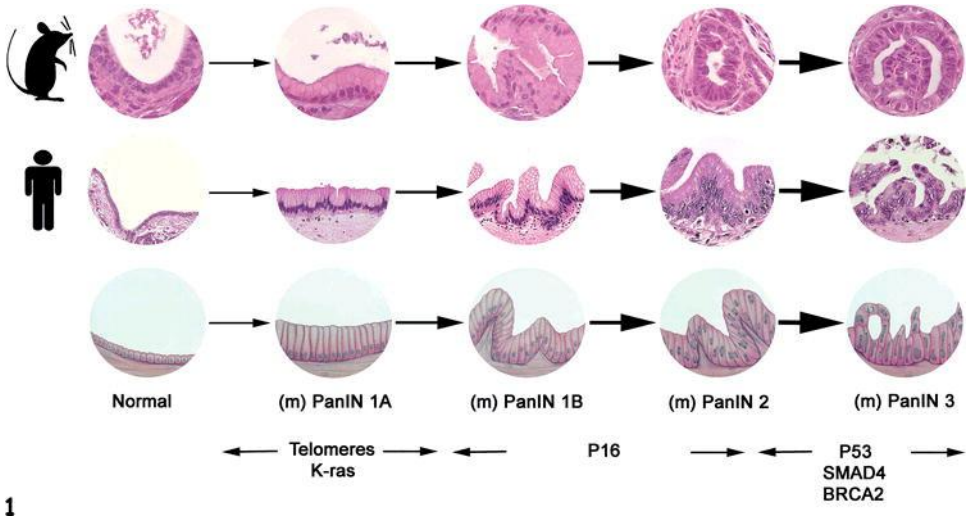
The poor prognosis of pancreatic cancer is mainly because the disease is almost always detected in a late stage, when advanced tumor growth exists and curative resection is no longer possible. Surgery is the only option for cure, but even when surgical resection is intended as a measure for complete removal of the malignancy, most patients will develop recurrence of the disease and will die due to metastatic growth [3–5].

Early detection, at a stage when there is still an option to cure, is at present the only way to improve the dismal outlook of patients diagnosed with pancreatic cancer. Recent advances in our understanding of pancreatic carcinogenesis may indeed translate to therapeutic options that will benefit pancreatic cancer patients. It is now well established that invasive pancreatic cancer develops through stepwise tumor progression and it is therefore preceded by preinvasive stages amenable to curative treatment. The precursor lesions of invasive pancreatic cancer are known as pancreatic intraepithelial neoplasia (PanIN) and the consecutive stages are morphologically well defined [6]. Pancreatic intraepithelial neoplasias are not the only precursors of invasive pancreatic cancer: Intraductal papillary mucinous neoplasms and mucinous cystic neoplasms, for example, are also preinvasive stages of carcinoma [7], but PanINs are the most common precursor of conventional ductal adenocarcinomas of the pancreas, and they are the subject of this review. Pancreatic tumor progression is not only morphologically relatively well defined but many of the molecular genetic alterations leading to pancreatic cancer are also known. Consecutive stages of tumor progression are accompanied by cumulative genetic disarray in which specific genetic alterations are the major players [8]. These specific genetic abnormalities are potential markers that enable early diagnosis and may become used to target intervention. Finally, recent genetically engineered mouse models of pancreatic cancer recapitulate the human disease quite accurately and these transgenic mouse models provide potentially interesting tools for translational research purposes [9].

Pancreatic intraepithelial neoplasia

It is now recognized that in the pancreas, ductal adenocarcinoma develops through a stepwise tumor progression model analogous to the adenoma-carcinoma sequence in the colorectum, in which consecutive preinvasive stages are relatively well defined and morphologically distinctive (Figure 1). These preinvasive lesions are designated PanINs and they are classified based on the degree of architectural and cytonuclear abnormalities in PanIN-1A or PanIN-1B, and PanIN-2 or PanIN-3

[6,7,10]. Pancreatic intraepithelial neoplasia lesions are found in the smaller pancreatic ducts (<5-mm diameter). In PanIN lesions the normal cuboidal flat epithelial lining of the ducts is replaced by columnar mucinous cells, either flat or papillary, with various degrees of dysplasia. Typically, PanIN-1A lesions contain a lining of flat mucinous epithelium and PanIN-1B lesions are covered by a papillary mucinous epithelium, in both cases with minimal cytonuclear atypia. In PanIN-2 lesions there is more cytonuclear atypia, pseudostratification, and loss of polarity consistent with low-grade dysplasia, whereas the features in PanIN-3 are those of high-grade dysplasia or carcinoma in situ with a more complex papillary architecture, nuclear hyperchromatism and pleomorphism, crowding, mitotic figures, and sometimes necrosis; the lesion is, however, still confined within the basement membrane and there is no invasive growth of tumor cells [6].



1

Figure 2 Tumor progression model of pancreatic carcinogenesis: bottom, schematic drawing; middle, in men; and top, in mice. The consecutive stages are formed by the various stages of pancreatic intraepithelial neoplasia lesions with their specific histopathology. During tumor progression there is an increasing generalized genetic disarray (reflected by the increasing thickness of arrows) in which alterations in specific oncogenes and tumor suppressor genes are the major players. The timing of these specific genetic events is represented at the bottom line.

Pancreatic intraepithelial neoplasias are proliferative lesions that are considered precursors of pancreatic ductal adenocarcinoma and are therefore neoplasms in the strict sense. The evidence that PanINs are indeed neoplastic lesions is derived from various sources and observations. Autopsy studies showed an increase of the prevalence of PanIN with age corresponding with the increase of pancreatic cancer rates in the older age groups [11]. In resection specimens with pancreatic ductal adenocarcinoma, PanINs are typically present in the area surrounding the tumor. PanIN-1 is found in 75%, PanIN-2 in 65%, and PanIN-3 in 50% of cases [12]. Well-

documented cases in which high-grade PanINs subsequently developed into invasive carcinoma have also been described [13].

Proteins that show an increased expression in ductal pancreatic carcinoma are also overexpressed in PanIN lesions. Cyclin D1 overexpression is associated with a poor prognosis in pancreatic cancer [14]. Nuclear cyclin D1 overexpression is not present in the normal pancreatic ducts or in PanIN-1 lesions. It is, however, seen in 29% of the PanIN-2 lesions and in 57% of the PanIN-3 lesions [8]. Cyclooxygenase 2 is a rate-limiting enzyme in the prostaglandin pathway and its overexpression is implicated in tumor cell growth, invasion, angiogenesis, and prognosis [15]. Cyclooxygenase 2 is upregulated in pancreatic cancer and overexpression is increasingly encountered in consecutive grades of PanIN [16]. Cyclooxygenase 2 overexpression in PanINs is particularly interesting because it is a potential target for chemotherapy by means of selective cyclooxygenase 2 inhibitors [17].

The strongest evidence that PanINs are precursors of pancreatic ductal carcinoma and truly neoplastic lesions comes, however, from molecular genetic studies in which the specific genetic alterations commonly encountered in pancreatic cancer were also found in PanINs, and, more importantly, these mutations were shown to accumulate during progression through the various stages of PanIN lesions.

Genetic alterations in PanINs

Cancer is a disease of the genes. An interplay between activated oncogenes and loss of function alterations in tumor suppressor genes leads to uncontrolled and autonomous tumor cell growth. Genetic instability makes the genome vulnerable to mutations in tumor suppressor genes and oncogenes and this instability increases during tumor progression. Genes responsible for the maintenance of the DNA integrity, such as DNA repair genes, can also be involved and their loss of function contributes to genetic alterations and instability of the genome. This genetic model for tumor growth is best known for colorectal tumorigenesis [18], but it holds true for pancreatic tumorigenesis as well, and in this regard pancreatic cancer is one of the better understood solid tumors [19].

The most common activating point mutation occurs in the *KRAS2* oncogene and more than 90% of the pancreatic cancers contain this mutation [20] (Figure 2, A). The mutation in pancreatic carcinomas always takes place in codon 12, and it is therefore easily detectable with simple molecular testing [21,22]. The *KRAS2* gene encodes a guanosine-5′-triphosphate binding protein with intrinsic guanosine-5′-triphosphatase activity. The guanosine-5′-triphosphatase activity is disrupted by the mutation, and as a result the protein remains constitutively active [21,22]. The protein regulates cell cycle progression via the mitogen-activated protein kinase and AKT pathways [23]. *KRAS2* mutations belong to the earliest genetic alterations observed in pancreatic tumorigenesis. *KRAS2* mutations are found in 10% to 30% of PanIN-1, 45% of PanIN-2, and 85% of PanIN-3 lesions [24].

Pancreatic intraepithelial neoplasia lesions may harbor the same mutation as the adjacent carcinoma, but this is not necessarily the case [25]. The high frequency of *KRAS2* mutations suggests that they can be considered an initiating event, and the genetically engineered animal models for pancreatic cancer support this notion: *KRAS2* mutation is a sine qua non for the development of ductal neoplasia in the mouse and the zebrafish [26,27]. Because in the human pancreas mutations always occur in the same codon, they can be detected in small samples [28]. These mutations are therefore a potentially interesting target for early diagnosis [21,22,29]. Nevertheless, false positivity is a concern because the mutations occur in the earliest lesions and the natural history of these lesions may not justify resection [30]. In high-risk patients and in combination with other high-resolution imaging methodology to visualize early lesions, *KRAS2*mutational analysis in cytology or pancreatic juice samples may have a role in diagnostic evaluation [31]. As a target for therapeutic intervention, the RAS signaling pathway has been disappointing so far [32].

In contrast to the dominantly acting oncogenes, tumor suppressor genes are recessive and, in general, both the paternal and the maternal copy need to be mutated to disrupt their function. Tumor suppressor genes inhibit tumor cell growth and disruption of their function contributes to tumorigenesis. The most important tumor suppressor genes that have a role in pancreatic tumorigenesis are *p16/INK4A, TP53,* and *DPC4/SMAD4.*
Virtually all pancreatic carcinomas harbor mutations in the *p16/INK4A* gene located on chromosome 9p. Homozygous deletion, intragenic mutation in combination with loss of the remaining allele, or hypermethylation of the promoter region constitute the various mechanisms of silencing of this gene in pancreatic carcinogenesis [33,34]. The gene encodes a cell cycle checkpoint protein that binds to Cdk4 and Cdk6 and in this fashion binding to cyclin D1 is prevented resulting in cell cycle arrest [35]. When there is loss of function of p16, inappropriate phosphorylation of Rb (retinoblastoma) leads to progress of the cell cycle and increased cellular proliferation [36]. The p16/Rb pathway is inactivated in 95% of the invasive pancreatic cancers, and absence of nuclear protein expression of p16, as assessed by immunohistochemistry, is found in 30%, 55%, and 70% of the invasive pancreatic cancer–associated PanIN-1, PanIN-2, and PanIN-3 lesions, respectively, indicating that loss of function is a relatively early event in pancreatic tumorigenesis [37]. This is also demonstrated by the fact that patients with a germline mutation in this gene, and particularly those with a *p16*-Leiden deletion, carry an increased risk for invasive pancreatic cancer [38,39] (Figure 2, B through D). In such patients early detection of precursor lesions through surveillance could be considered and may lead to a decision of prophylactic surgery [38].

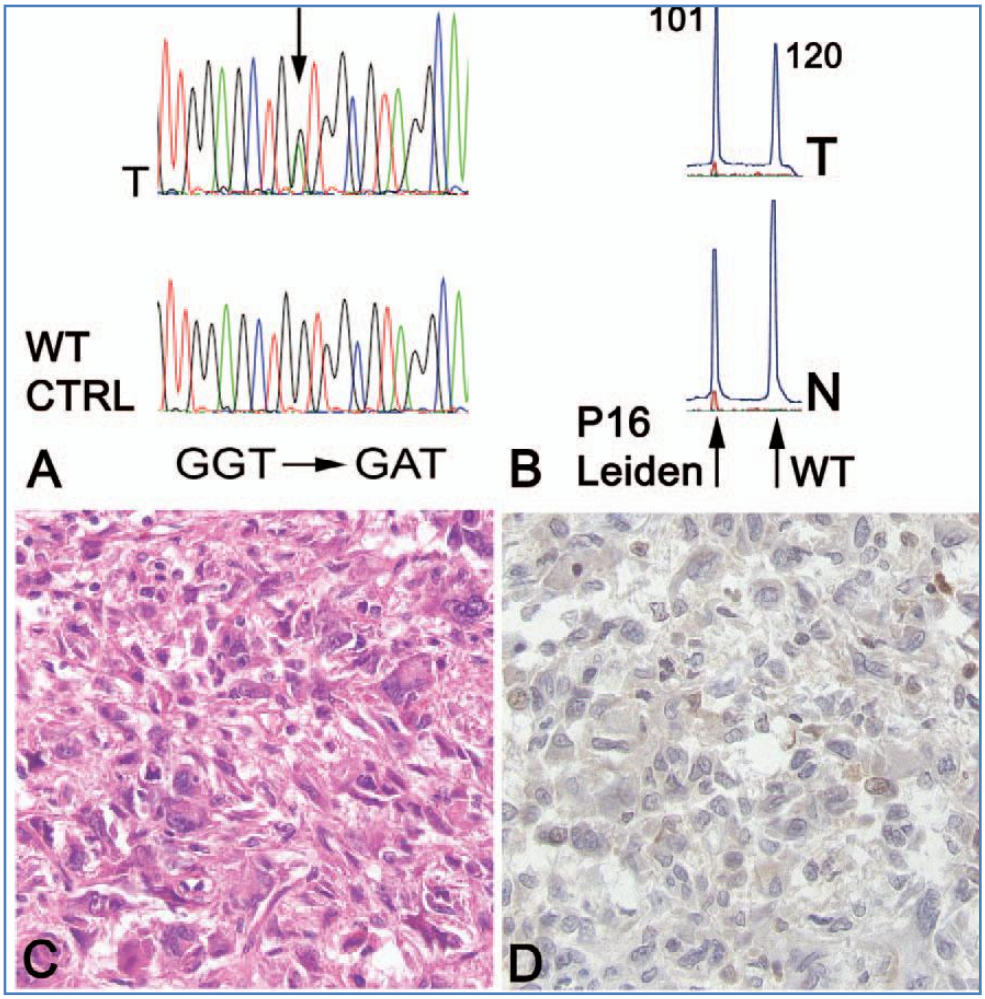


Figure 3 A, Sequence analysis of KRAS2 mutation in pancreatic cancer of patient with germline p16 mutation. B, NA profile of p16 in the carcinoma: left peak is allele with germline mutation (p16-Leiden deletion), right peak is the remaining lele that is retained in the noncancerous tissue (lower) but lost in the cancer (upper, 40% reduction in peak height). C, carcinoma (hematoxylin-eosin, original magnification x200). D, p16 immunostaining shows no expression of the p16 protein product in the tumor cells; a few inflammatory cells stained positive (original magnification x200).

Homozygous deletions of *p16* may encompass adjacent genes. *MTAP* is such a gene and it is located 100 kilobases telomeric to the *p16/INK4A* gene [40] Therefore, this gene is regularly lost with deletions of *p16.* This may have therapeutic implications as the MTAP protein product plays a role in the biosynthesis of adenosine, and chemotherapeutic agents have been developed to specifically target the *MTAP* loss in pancreatic neoplasms [40].

The *TP53* tumor suppressor gene, located on chromosomal arm 17p, is the most frequently inactivated tumor suppressor gene in human solid neoplasms, and it is also inactivated in 55% to 75% of the invasive pancreatic cancers. The protein product acts as a transcription factor that binds to DNA and when mutated, this binding is disrupted [41]. The protein has a number of critically important functions, most notably as a checkpoint protein that enables repair of DNA damage during the cell cycle arrest or as a protein that induces apoptosis when the DNA damage is irreparable [42]. In the pancreas, loss of functional p53 typically occurs through an intragenic mutation in combination with loss of the remaining wild-type allele [43]. *TP53* mutations generally lead to stabilization of the protein product and positive nuclear staining with immunohistochemistry [44]. Positive immunostaining is therefore an accurate surrogate marker for mutations [44].

Positive p53 staining is typically seen in PanIN-3 lesions, that is at the transition from in situ carcinoma to invasive pancreatic cancer [8] (Figure 3, A and B). *TP53* mutations are a relatively late event in pancreatic tumorigenesis. Positive p53 immunostaining may nevertheless be of value in the early detection of neoplasia by means of brush cytology in high-risk patients [45].

Another tumor suppressor gene that is frequently inactivated in invasive pancreatic cancer is *DPC4* or *SMAD4* located on chromosomal arm 18q. DPC stands for deleted in invasive pancreatic cancer and indeed this genetic alteration is quite specific for pancreas carcinomas, although it can occasionally be encountered in colon, breast, ovarian, and bile duct cancers as well [46,47]. The available antibodies against the DPC4/SMAD4 protein are highly suitable for immunohistochemistry and expression accurately mirrors the gene status [48]. Absence of nuclear expression in immunohistochemical stainings can therefore be of diagnostic value [49]. Loss of function is generally caused by a homozygous deletion or an intragenic mutation associated with loss of the second allele in invasive pancreatic cancer and 55% of the pancreatic carcinomas harbor such alterations [47]. The DPC4/SMAD4 protein is an intermediate in the transforming growth factor β signaling pathway. On transforming growth factor β/activin receptor activation, SMAD2 and SMAD3 proteins are phosphorylated and heterodimerize with the SMAD4 protein [50]. Thereafter the complex translocates to the nucleus where SMAD4 acts as a transcription factor and transactivates target genes involved in growth inhibition and apoptosis [50]. Like *TP53*, mutations in *DPC4/SMAD4* occur relatively late during pancreatic tumorigenesis and are only seen in PanIN-3 lesions (30%–40%) and invasive cancers [49] (Figure 3, C and D).

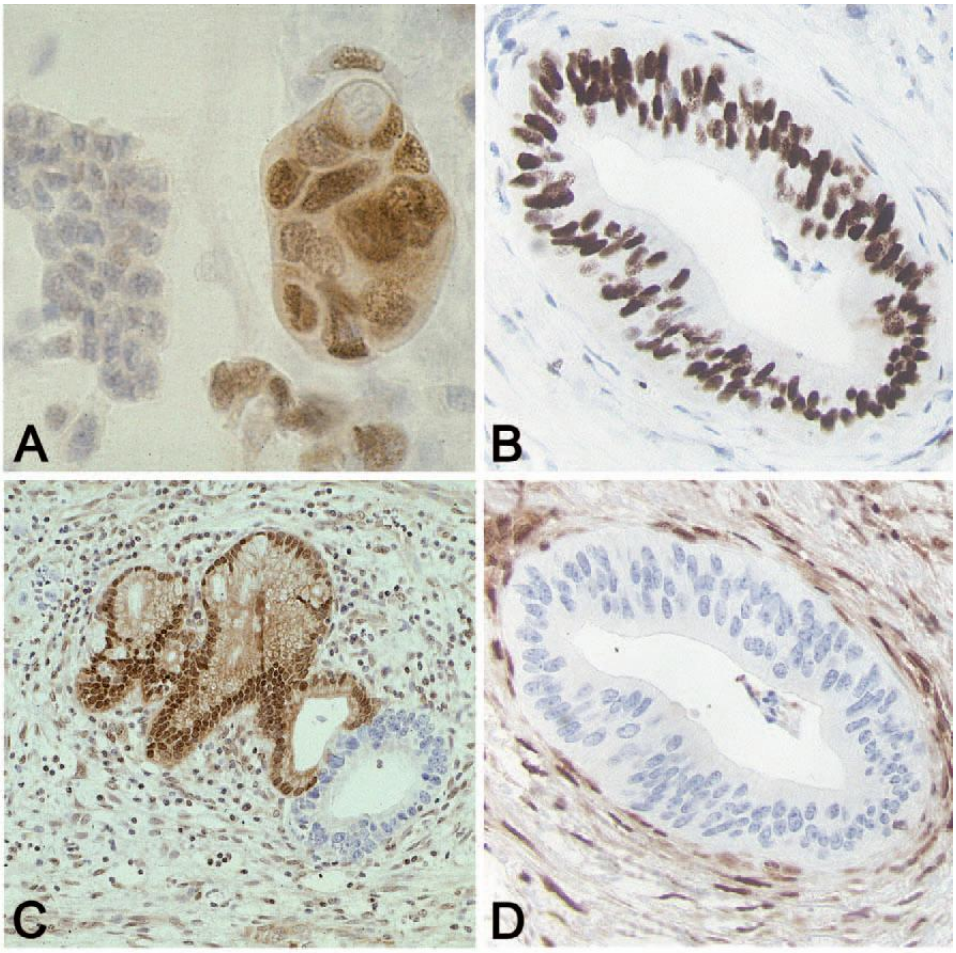


Figure 4 A, Positive p53 immunostaining in brush cytology obtained during endoscopic retrograde cholangiopancreatography of patient with pancreatic cancer (original magnification x630). B, Positive p53 immunostaining in pancreatic intraepithelial neoplasia 3 lesion (original magnification x400). C, Negative immunostaining of DPC4 in carcinoma adjacent to positively staining duct (original magnification x100). D, Negative DPC4 staining in pancreatic intraepithelial neoplasia 3 (same as in B) (original magnification x400).

Loss of function of genes that play a role in DNA repair and in maintaining the integrity of the genome also contribute to pancreatic carcinogenesis. This includes among others the Fanconi anemia family of genes. Fanconi anemia is a hereditary cancer susceptibility syndrome and affected family members usually die at a young age due to hematologic malignancies [51]. Those who survive develop solid tumors at an older age. The genes of the Fanconi anemia pathway involved in pancreatic tumorigenesis include the *FANCC* and *FANCG* genes and most notably the *BRCA2* gene [52]. The *BRCA2* gene product plays a role in DNA repair of so-called DNA-interstrand cross-links through homologous recombination

[53]. Because these DNA-interstrand cross-links are deliberately introduced by certain chemotherapeutic agents, such as cis-platinum and mitomycin, it is likely that pancreatic neoplasms with disruption of the Fanconi anemia pathway may be more susceptible to such chemotherapeutic agents [53]. The percentage of invasive pancreatic cancers with *BRCA2* mutations is less than 10% [54]. *BRCA2* germline mutation is accompanied by increased risk for invasive pancreatic cancer and a founder mutation exists among the Ashkenazi Jewish population [55]. In patients with invasive pancreatic cancer known to carry a *BRCA2* germline mutation, loss of the second allele was found in a PanIN-3 lesion but not in the low-grade lesions, indicating that complete loss of function of *BRCA2* is a late event comparable with the timing of loss of function of *TP53* and *SMAD4/DPC4* [56].

Mismatch repair genes encode proteins that form complexes that act as repair systems for replication errors occurring during DNA replication, particularly in DNA nucleotide repeat tracks. These errors, if not repaired, introduce so-called microsatellite instability into the genome and predispose to tumorigenesis [57]. Defects in the mismatch repair genes are rare in pancreatic tumorigenesis and less than 5% of invasive pancreatic cancers contain such mutations. When defects in the mismatch repair system occur, they are accompanied by microsatellite instability and medullary type carcinomas [58]. Similar to colorectal cancer, these tumors have a better prognosis than conventional ductal adenocarcinomas of the pancreas, and *KRAS2* mutations are not found [57,58].

It is thought that the earliest event leading to genomic instability in pancreatic carcinogenesis involves a telomeric crisis initiated by erosion of the telomeres [59]. Telomeres are present at the end of the chromosomes and consist of specific DNA sequence repeats bound to proteins. These caps on the chromosomal ends protect against fusion of the chromosomes. Extreme shortening of the telomeres leads to the formation of anaphase bridges during mitosis and consequently to numerical and structural instability of the genome [60]. Telomere erosion occurs during the early stages of pancreatic tumorigenesis and is seen in more than 90% of the lowest grade PanIN lesions [59]. It is believed that the chromosomal instability due to this telomeric crisis may be the underlying cause of further genetic disarray, which develops during tumor progression in most invasive pancreatic cancers [59,60].

Genically engineered mouse models

Genetically engineered mouse models for invasive pancreatic cancer make use of the transduction of genetically manipulated DNA into the developing animal and it is necessary to refer briefly to pancreatic embryology to provide a framework for the understanding of the findings from transgenic mouse models. The pancreas is formed by the dorsal and ventral pancreatic buds from foregut endoderm. After

fusion of the buds, the proximal duct of the ventral bud and the distal duct of the dorsal bud fuse to form the main pancreatic duct in humans. Formation of the dorsal and ventral bud, subsequent branching morphogenesis, and cytodifferentiation in specialized endocrine and exocrine cells is governed by a transcriptional machinery in which activation and repression of specific transcription factors dictate an orderly organogenesis [61]. The dorsal bud develops through inhibition of Hedgehog signaling in a specific region of the endoderm, whereas the default development of the ventral bud is toward pancreas and here the induction of Hedgehog signaling limits the extent of the ventral bud formation [62,63]. Early morphogenesis of the pancreas is governed by 3 transcription factors: Pdx1, HlxB9, and p48 [64]. Homozygous deletion of Pdx1 leads to pancreatic agenesis [64]. Loss of HlxB9 expression leads to agenesis of the dorsal but not the ventral bud [65]. The pancreatic epithelium contains all the precursor cells of the mature organ and differentiates along islet cell, acinar cell, and ductal cell lineages giving rise to the mature organ. Cells committed to the exocrine cell lineage lose Pdx1 expression but maintain p48 expression. Islet cell precursors maintain Pdx1 expression, lose p48 expression, and acquire neurogenin 3 expression [66,67]. An important role in the development and maturation of the pancreas is played by Notch. Notch signaling inhibits p48, and loss of Notch signaling permits differentiation into acinar cells [68,69]. Notch expression serves to maintain a pool of undifferentiated cells in the mature organ [69]. Also, various components of the notch signaling pathway, which are extinguished in the mature organ, are aberrantly reactivated in PanIN lesions and invasive pancreatic cancer [70]. In vitro Notch induction induces acinar to ductal metaplasia, whereas γ secretase inhibitors (inhibiting Notch) have the opposite effect [70]. Similarly, components of the Hedgehog signaling pathway are upregulated during pancreatic tumorigenesis, whereas no expression occurs in the mature normal pancreas [71]. Cyclopamine, an inhibitor of Hedgehog, reduced tumor mass of xenografted invasive pancreatic cancers in nude mice [71]. These numerous observations support the notion that pancreatic tumorigenesis appears to be associated with reactivation of embryonic pathways that play a role in developmental morphogenesis, similar to (tumorigenesis in) other compartments of the digestive tract [72].

Various genetically engineered mouse models of invasive pancreatic cancer have been developed and the pathology of most of them has been critically evaluated, with special emphasis on the similarities and differences with respect to human disease [73].

Mutant *KRAS* endogenously expressed in the developing murine pancreas is a prerequisite for the development of ductal type adenocarcinomas [26], and only these mouse models developed bona fide mouse pancreatic intraepithelial neoplasia (mPanIN) lesions ranging from low to high grade. Hingorani et al [26] developed a model in which expression of an oncogenic $KRAS^{G12D}$ allele is activated by a *Pdx1-Cre* transgene. The Pdx1-Cre, $Kras^{G12D}$ mice develop mPanIN of various degrees and some develop ductal type invasive cancers as well, thereby

faithfully recapitulating the natural history of the human disease (Figure 1). Not only do these mPanIN lesions resemble the human lesions microscopically, but they also overexpress proteins such as Notch, Hedgehog, and cyclooxygenase 2, similar to the human situation [26]. When crossed with a biallelic *INK4A/Arf* deletion or an oncogenic *Trp53*^{R172H} allele, these mice develop more aggressive tumors with metastasis, short latency, and full penetrance [74,75]. Importantly, sole abrogation of *INK4A/Arf* or *TP53,* without mutant *KRAS*, does not lead to pancreatic carcinogenesis [74,75].

The development of the genetically engineered mouse models of ductal type invasive pancreatic cancer has enhanced our understanding of the disease considerably. The mouse models have confirmed the importance of mutantly activated *KRAS* in ductal pancreatic adenocarcinogenesis. The fact that the carcinomas are also accompanied by low- and high-grade mPanINs in these mouse models, microscopically resembling their human counterparts, is yet another argument in favor of their role as the preceding noninvasive precursor stages of ductal pancreatic adenocarcinoma and supports the legitimacy of the postulated human tumor progression model.

Interestingly, despite the previous bona fide mouse models of human pancreatic carcinogenesis, the earliest lesion and initiating event, that is the cell of origin of invasive pancreatic cancer, remains to be elucidated. Given their ultimate ductal phenotype and because the precursor lesions also exhibit features of ductal differentiation, one would perhaps assume that the ductal epithelial cells give rise to the carcinomas. Surprisingly, targeting of oncogenic *KRAS* into the mature ductal epithelium does not induce neoplastic growth in the murine pancreas [76]. Growth of mPanINs and ductal adenocarcinomas can be produced in the adult pancreas of mice by conditional expression of mutant *KRAS* in the acinar/centroacinar compartment, but only if accompanied by ongoing injury and chronic inflammation [76]. These findings implicated the acinar/centroacinar compartment as the site of origin of ductal pancreatic adenocarcinoma and they underscored the importance of chronic pancreatitis as a risk factor for cancer, a long-standing well-known fact from epidemiologic studies [77]. These observations also led to a critical reappraisal of the morphologic findings in specimens in which chronic inflammation surrounds the pancreatic carcinoma, with particular attention to foci of so-called acinar to ductal metaplasia [73]. This metaplasia is a common finding in a variety of genetically engineered mouse models and is characterized by tubular structures that have features of acinar as well as ductular differentiation [77]. It is also one of the first abnormalities seen after ligation of the pancreatic duct in other animal models [78]. In the human pancreas, acinar to ductal differentiation is particularly seen in the setting of chronic pancreatitis and these lesions can be associated with PanINs [79]. From the previous mouse model in which there is induction of chronic injury, it appears that the chronic inflammation resulting in acinar to ductal metaplasia causes otherwise quiescent cells to adapt a progenitor-like cell type, susceptible to oncogenic transformation by mutant *KRAS* activation;

this could be due to dedifferentiation of mature acinar cells, expansion of a centroacinar progenitor cells, or both [77]. As mentioned earlier, in vitro systems show a similar phenomenon after Notch activation, and the observed reactivation of Notch pathway components in PanIN lesions is of particular interest in this context [70]. In an attempt to integrate all these data into pathologic findings in human disease, a recent study evaluated resection specimens harboring acinar to ductal metaplasia accompanied by PanIN lesions and performed *KRAS2* mutational analysis. Mutations were not found in acinar cells or isolated foci of acinar to ductal metaplasia, that is cells distant from the PanIN lesions. Of the metaplastic foci associated with a PanIN lesion, one-quarter harbored a *KRAS2* mutation, and this mutation was identical to the one in the accompanying PanIN lesion. In the remaining PanIN lesions with a *KRAS2* mutation, the associated acinar to ductal metaplasia did not contain this *KRAS2* mutation [80]. Under the assumption that *KRAS2* mutation is the earliest genetic event in pancreatic carcinogenesis, the findings do not seem to support the conclusion drawn from the mice studies that dedifferentiated acinar cells or centroacinar progenitor cells are the putative cell of origin for human ductal pancreatic adenocarcinoma.

Conclusions

Ductal pancreatic adenocarcinoma is the most common cancer of the pancreas and a lethal disease; early detection, in a stage when surgical resection is still an option for cure, is currently the only hope for a better outcome. The noninvasive precursor stages of ductal pancreatic adenocarcinoma, called PanINs, are well-defined and potential targets for early diagnosis or treatment. Genetically engineered mouse models for ductal pancreatic carcinoma are an important adjunct to the armamentarium of translational research. These models faithfully recapitulate the natural course and biology of the human disease. In both mice and men, pancreatic cancer appears to be initiated by oncogenic *KRAS2.* Inactivation of various tumor suppressor genes then accelerates tumor progression in the mouse and the human exocrine pancreas, including *TP53, P16/INK4A*, and *SMAD4* or transforming growth factor β receptor 2. Interestingly, recent evidence suggests that the cell origin of human pancreatic carcinogenesis may differ from that in the mouse.

Reference list

1. Jemal A, Siegel R and Ward E, *Cancer statistics*, 2006. CA Cancer J Clin 2006. 56:106-130
2. American Cancer Society, *Cancer facts & figures 2007*. Atlanta, GA: American Cancer Society; 2007:1–52.
3. Sohn TA, Yeo CJ, Cameron JL et al., *Resected adenocarcinoma of the pancreas—616 patients: results, outcomes, and prognostic indicators.* J Gastrointest Surg 2000. 4:567–579.
4. Winter JM, Cameron JL, Campbell KA et al., *1423 pancreatico-duodenectomies for pancreatic cancer: a single-institution experience.* J Gastrointest Surg 2006. 10:1199–1210.
5. Li D, Xie K, Wolff R, et al., *Pancreatic cancer.* Lancet 2004. 363:1049–1057.
6. Hruban RH, Adsay NV, Albores-Saavedra J, et al., *Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions.* Am J Surg Pathol 2001. 25:579–586.
7. Hruban RH, Takaori K, Klimstra DS, et al., *An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms.* Am J Surg Pathol 2004. 28:977–987.
8. Maitra A, Adsay NV, Argani P, et al., *Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray.* Mod Pathol 2003. 16:902–912.
9. Leach SD, *Mouse models of pancreatic cancer: the fur is finally flying!* Cancer Cell 2004. 5:7–11.
10. Hruban RH, Klimstra DS, and Pitman MB, *Tumors of the Pancreas.* Washington, DC: Armed Forces Institute of Pathology; 2007. Atlas of Tumor Pathology; 4th series, fascicle 6.
11. Cubilla AL and Fitzgerald PJ, *Morphological lesions associated with human primary invasive nonendocrine pancreas cancer*.Cancer Res 1976. 36:2690–2698.
12. Andea A, Sarkar F, Adsay VN, *Clinicopathological correlates of pancreatic intraepithelial neoplasia: a comparative analysis of 82 cases with and 152 cases without pancreatic ductal adenocarcinoma.* Mod Pathol 2003. 16:996–1006.
13. Brat DJ, Lillemoe KD, Yeo CJ, et al., *Progression of pancreatic intraductal neoplasias to infiltrating adenocarcinoma of the pancreas.* Am J Surg Pathol 1998. 22:163–169.
14. Kornmann M, Ishiwata T, Itakura J, P. et al., *Increased cyclin D1 in human pancreatic cancer is associated with decreased postoperative survival.* Oncology 1998. 55:363–369.
15. van Rees BP and Ristimäki A. *Cyclooxygenase-2 in carcinogenesis of the gastrointestinal tract.* Scand J Gastroenterol 2001. 36:897–903.
16. Maitra A , Ashfaq R, Gunn CR, et al., *Cyclooxygenase 2 expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasia: an immunohistochemical analysis with automated cellular imaging*. Am J Clin Pathol 2002. 118:194–201.
17. Keller JJ and Giardiello FM. *Chemoprevention strategies using NSAIDs and COX-2 inhibitors.* Cancer Biol Ther 2003. 2:140S–149S.
18. Kinzler KW and Vogelstein B, *Lessons from hereditary colorectal cancer.* Cell 1996. 87:159–170.
19. Hruban RH, Goggins M, Parsons J, et al., *Progression model for pancreatic cancer.* Clin Cancer Res 2000. 6:2969–2972.
20. Hruban RH , van Mansfeld AD, Offerhaus GJA. et al. *K-ras oncogene activation in adenocarcinoma of the human pancreas: a study of 82 carcinomas using a*

combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. Am J Pathol 1993. 143:545–554.
21. Sturm PD, Hruban RH, Ramsoekh TB, et al., *The potential diagnostic use of K-ras codon 12 and p53 alterations in brush cytology from the pancreatic head region.* J Pathol 1998. 186:247–253.
22. Tascilar M, Caspers E, Sturm PD, et al., *Role of tumor markers and mutations in cells and pancreatic juice in the diagnosis of pancreatic cancer*. Ann Oncol 1999. 10:(suppl 4). 107–110.
23. Hingorani SR and Tuveson DA, *Ras redux: rethinking how and where Ras acts.* Curr Opin Genet Dev 2003. 13:6–13.
24. Löhr M, Klöppel G, Maisonneuve P, et al., *Frequency of K-ras mutations in pancreatic intraductal neoplasias associated with pancreatic ductal adenocarcinoma and chronic pancreatitis: a meta-analysis.* Neoplasia 2005. 7:17–23.
25. Lüttges J, Diederichs A, Menke MA, et al., *Ductal lesions in patients with chronic pancreatitis show K-ras mutations in a frequency similar to that in the normal pancreas and lack nuclear immunoreactivity for p53.* Cancer2000. 88:2495–2504.
26. Hingorani SR , Petricoin EF, Maitra A, et al., *Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse.* Cancer Cell 2003. 4:437–450.
27. WooPark S , Davison JM, Rhee J, et al., *Oncogenic KRAS induces progenitor cell expansion and malignant transformation in zebrafish exocrine pancreas*. Gastroenterology 2008. 134:2080–2090.
28. van Es JM , Polak MM, van den Berg FM, et al., *Molecular markers for diagnostic cytology of neoplasms in the head region of the pancreas: mutation of K-ras and overexpression of the p53 protein product*. J Clin Pathol 1995. 48:218–222.
29. Sturm PD , Rauws EA, Hruban RH, et al., *Clinical value of K-ras codon 12 analysis and endobiliary brush cytology for the diagnosis of malignant extrahepatic bile duct stenosis.* Clin Cancer Res 1999. 5:629–635.
30. van Heek NT, Rauws EA, Caspers E, et al., *Long-term follow-up of patients with a clinically benign extrahepatic biliary stenosis and K-ras mutation in endobiliary brush cytology.* Gastrointest Endosc 2002. 55:883–888.
31. Brentnall TA , Bronner MP, Byrd DR, et al., *Early diagnosis and treatment of pancreatic dysplasia in patients with a family history of pancreatic cancer.* Ann Intern Med 1999. 131:247–255.
32. Karp JE and Lancet JE, *Development of farnesyltransferase inhibitors for clinical cancer therapy: focus on hematologic malignancies.* Cancer Invest 2007. 25:484–494.
33. Caldas C , Hahn SA, da Costa LT, et al., *Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma.* Nat Genet 1994. 8:27–32.]
34. Schutte M, Hruban RH, Geradts J, et al., *Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas.* Cancer Res 1997. 57:3126–3130.
35. Sherr CJ, *Cell cycle control and cancer.* Harvey Lect 2000–2001. 96:73–92.
36. Sellers WR, Rodgers JW, and Jr Kaelin WG, *A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites.* Proc Natl Acad Sci U S A 1995. 92:11544–11548.
37. Wilentz RE, Geradts J, Maynard R, et al., *Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression.* Cancer Res 1998. 58:4740–4744.

38. de Vos tot Nederveen Cappel WH, Offerhaus GJA, van Puijenbroek M, et al., *Pancreatic carcinoma in carriers of a specific 19 base pair deletion of CDKN2A/p16 (p16-leiden).* Clin Cancer Res 2003. 9:3598–3605.
39. Koorstra JB, Hustinx SR, Offerhaus GJA, et al., *Pancreatic carcinogenesis.* Pancreatology 2008. 8:110–125.
40. Hustinx SR, Leoni LM, Yeo CJ, et al., *Concordant loss of MTAP and p16/ CDKN2A expression in pancreatic intraepithelial neoplasia: evidence of homozygous deletion in a noninvasive precursor lesion.* Mod Pathol 2005. 18:959–963.
41. Kern SE, Kinzler KW, Bruskin A, et al., *Identification of p53 as a sequence-specific DNA-binding protein.* Science 1991. 252:1708–1711.
42. Kern SE, *p53: tumor suppression through control of the cellycle.* Gastroenterology 1994. 106:1708–1711.
43. Redston MS, Caldas C, Seymour AB, et al., *p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions*. Cancer Res 1994. 54:3025–3033.
44. Baas IO, Mulder JW, Offerhaus GJA, et al., *An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms.* J Pathol 1994. 172:5–12.
45. Tascilar M, Sturm PD, Caspers E, et al., *Diagnostic p53 immunostaining of endobiliary brush cytology: preoperative cytology compared with the surgical specimen.* Cancer 1999. 87:306–311.
46. Schutte M, Hruban RH, Hedrick L, et al., *DPC4 gene in various tumor types.* Cancer Res 1996. 56:2527–2530. [
47. Hahn SA , Schutte M, Hoque AT, et al., *DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1*.Science 1996. 271:350–353.
48. Wilentz RE , Su GH, Dai JL, et al., *Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas: a new marker of DPC4 inactivation.* Am J Pathol 2000. 156:37–43.
49. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al., *Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression.* Cancer Res 2000. 60:2002–2006.
50. Blobe GC, Schiemann WP, and Lodish HF, *Role of transforming growth factor beta in human disease.* N Engl J Med 2000. 342:1350–1358.
51. van der Heijden MS, Yeo CJ, Hruban RH, et al., *Fanconi anemia gene mutations in young-onset pancreatic cancer.* Cancer Res 2003. 63:2585–2588.
52. van der Heijden MS, Brody JR, Gallmeier E, et al., *Functional defects in the Fanconi anemia pathway in pancreatic cancer cells.* Am J Pathol 2004. 165:651–657.
53. van der Heijden MS, Brody JR, Dezentje DA, et al., *In vivo therapeutic responses contingent on Fanconi anemia/BRCA2 status of the tumor.* Clin Cancer Res 2005. 11:7508–7515. [Medline]
54. Goggins M, Schutte M, Lu J, et al., *Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas.* Cancer Res 1996. 56:5360–5364.
55. Hahn SA , Greenhalf B, Ellis I, et al., *BRCA2 germline mutations in familial pancreatic carcinoma.* J Natl Cancer Inst 2003. 95:214–221. [Medline]
56. Goggins M, Hruban RH, and Kern SE, *BRCA2 is inactivated late in the development of pancreatic intraepithelial neoplasia: evidence and implications.* Am J Pathol 2000. 156:1767–1771.
57. Goggins M, Offerhaus GJA, Hilgers W, et al., *Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology: poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+.* Am J Pathol 1998. 152:1501–1507.

58. Wilentz RE, Goggins M, Redston M, et al., *Genetic, immunohistochemical, and clinical features of medullary carcinoma of the pancreas: a newly described and characterized entity.* Am J Pathol 2000. 156:1641–1651.
59. van Heek NT, Meeker AK, Kern SE, et al., *Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia.* Am J Pathol 2002. 161:1541–1547.
60. Maitra A, Kern SE, and Hruban RH, *Molecular pathogenesis of pancreatic cancer.* Best Pract Res Clin Gastroenterol 2006. 20:211–226.
61. Kim SK and MacDonald RJ, *Signaling and transcriptional control of pancreatic organogenesis*. Curr Opin Genet Dev 2002. 12:540–547.
62. Hebrok M, Kim SK, and Melton DA, *Notochord repression of endodermal Sonic hedgehog permits pancreas development*. Genes Dev 1998. 12:1705–1713.
63. Hebrok M, Kim SK, St Jacques B, et al., *Regulation of pancreas development by hedgehog signaling.* Development 2000. 127:4905–4913.
64. Offield MF, Jetton TL, Labosky PA, et al., *PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum.* Development 1996. 122:983–995.
65. Li H, Arber S, Jessell TM, et al., *Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9*. Nat Genet 1999. 23:67–70.
66. Kawaguchi Y, Cooper B, Gannon M, M, et al., *The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors*. Nat Genet 2002. 32:128–134.
67. Schwitzgebel VM, *Programming of the pancreas*. Mol Cell Endocrinol 2001. 185:99–108.
68. Apelqvist A, Li H, Sommer L, et al., *Notch signalling controls pancreatic cell differentiation*. Nature 1999. 400:877–881.
69. Esni F, Ghosh B, Biankin AV, et al., *Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas*. Development 2004. 131:4213–4224.
70. Miyamoto Y, Maitra A, Ghosh B, et al., *Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis.* Cancer Cell 2003. 3:565–576.
71. Berman DM, Karhadkar SS, Maitra A, et al., *Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours.* Nature 2003. 425:846–851.
72. van den Brink GR and Offerhaus GJ. *The morphogenetic code and colon cancer development*. Cancer Cell 2007. 11:109–117.
73. Hruban RH, Adsay NV, Albores-Saavedra J, et al., *Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations.* Cancer Res 2006. 66:95–106.
74. Aguirre AJ, Bardeesy N, Sinha M, et al., *Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma.* Genes Dev 2003. 17:3112–3126.
75. Hingorani SR, Wang L, Multani AS, et al., *Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice.* Cancer Cell 2005. 7:469–483.
76. Guerra C, Schuhmacher AJ, Cañamero M, et al., *Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice.* Cancer Cell 2007. 11:291–302.
77. Murtaugh LC and Leach SD, *A case of mistaken identity?: nonductal origins of pancreatic "ductal" cancers*. Cancer Cell 2007. 11:211–213.
78. Boerma D, Straatsburg IH, Offerhaus GJ, et al., *Experimental model of obstructive, chronic pancreatitis in pigs.* Dig Surg 2003. 20:520–526.

79. Brune K, Abe T, Canto M, et al., *Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer*. Am J Surg Pathol 2006. 30:1067–1076.
80. Shi C, Hong SM, Lim P, et al., *KRAS2 gene mutations in human pancreatic acinar-ductal metaplastic lesions are limited to those associated with pancreatic intraepithelial neoplasia.* Mol Cancer Res. 2009. In press.

Chapter 4

Molecular characteristics of pancreatic ductal adenocarcinoma

Niki A. Ottenhof, Roeland F. de Wilde, Anirban Maitra, Ralph H. Hruban, G. Johan A. Offerhaus

Pathology Research International 2011; 620601

Abstract

Pancreatic cancer is an almost universally lethal disease and despite extensive research over the last decades, this has not changed significantly. Nevertheless, much progress has been made in understanding the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) suggesting that different therapeutic strategies based on these new insights, are forthcoming. Increasing focus exists on designing so-called targeted treatment strategies in which the genetic characteristics of a tumor guide therapy. In the past, the focus of research was on identifying the most frequently affected genes in PDAC, but with the complete sequencing of the pancreatic cancer genome the focus has shifted to defining the biological function that the altered genes play. For this review we aimed to put the genetic alterations present in pancreatic cancer in the context of their role in signaling pathways. In addition, this review provides an update of the recent advances made in the development of the targeted treatment approach in PDAC.

Pancreatic ductal adenocarcinoma

Annually, approximately 43,140 people are diagnosed (incidence 10-12:100,000) with pancreatic ductal adenocarcinoma (PDAC) in the Unites States and the mortality rate of 36,800, almost equals this number [1]. PDAC ranks fourth on the list of cancer-related causes of death and despite extensive clinical and scientific effort, the prognosis of this exceptionally lethal disease has not improved significantly over the past decades. Surgical resection, for which only a minority (<20%) of patients qualify due to advanced stage of disease at time of diagnosis, is currently the only chance for cure, improving five-year survival rates from <4% if left untreated to 25-30% after resection [2-4]. Though of marginal impact, chemo(radiation) therapy administered in (neo)adjuvant setting has been shown to increase short-term survival rates in resectable and advanced stage disease. [5-7]. Despite subtle progress over the years in terms of therapeutic strategies, no major new treatment options have come forward from numerous clinical trails. Nevertheless, much progress has been made in understanding the pathogenesis of PDAC during the past decades, suggesting that different therapeutic strategies based on these new insights, are on the horizon [8-10].

PDAC, like all cancers, is fundamentally a genetic disease caused by alterations in cancer-associated genes. The identification of such specific mutated genes is critical for understanding the pathogenesis of PDAC. Nevertheless, one cannot achieve a reasonable overview by considering only individual genes in a cancer cell because the neoplastic potential of this cell is the end product of mutations in multiple genes and changes in multiple pathways that interact and reinforce each other. The rapidly expanding knowledge of genetic and molecular alterations and their role in pancreatic carcinogenesis has led to the question whether it is possible to design a patient-specific therapy based on the genetic fingerprint of an individual tumor. Since an increasing focus exists on designing these so-called targeted treatment strategies, this review is aimed to put genetic alterations pancreatic cells undergo during malignant transformation in the context of their role in signaling pathways. In addition, this review provides an update of the most recent advances made in the development of the targeted treatment approach in PDAC.

Precursors of PDAC

The development of invasive carcinoma in the pancreas is a stepwise process. Similar to colon cancer, non-invasive stages have been identified in PDAC preceding invasive carcinoma [11]. In recently published research, the clonal evolution of the earliest genetic alterations in tumor initiating cells towards frankly invasive and metastasized PDAC was followed and these studies indicated that such tumor progression takes at least more than a decade [12]. This creates an

important window of opportunity for early detection and much effort is put into attempts to map the genetic changes that take place in the pancreatic ductal cells of precursor lesions before they become invasive.

Since 2004, there have been clear guidelines for classifying these precursor lesions of PDAC and three different types have been identified: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasia (MCN) and intraductal pancreatic mucinous neoplasia (IPMN) [13]. MCN and IPMN are considered separate and specific entities that fall beyond the scope of this review [14, 15]. By far the most common and also the generic precursor lesion of PDAC is the PanIN lesion. PanINs are found in the smaller pancreatic ducts and based on the degree of dysplasia reflected in the cytonuclear atypia and architectural change can be classified in four grades: PanIN-1A, PanIN-1B, PanIN-2 and PanIN-3. The least severe abnormalities are seen in PanIN-1 lesions; minimal cytonuclear atypia is present and cell polarity is retained with a basally located nucleus. The difference between PanIN-1A and -1B is that the cells in PanIN-1A lesions are flat whereas the cells in PanIN-1B lesions are arranged in a micropapillary architecture. PanIN-2 lesions are characterized by evident cytonuclear atypia and infrequent mitoses. PanIN-3 lesions, also called carcinoma-*in-situ*, demonstrate all of the hallmarks of cancer including loss of polarity, nuclear atypia, frequent mitoses, and budding of groups of cells in the lumen. Yet, the lesion is confined within the basement membrane and no invasive growth is present [13]. The increasing grades of dysplasia in the various PanIN lesions manifest the morphological steps of tumor progression that precede invasive PDAC. These consecutive steps of tumor progression are genetically accompanied by a cumulative occurrence of specific and generalized molecular genetic alterations. Typically, an interplay between mutations in tumor-suppressor genes, oncogenes and genome maintenance genes ultimately results in the development of PDAC.

Telomere shortening is considered the initiating event in pancreatic tumorigenesis by inducing genetic instability, and is discussed separately below. Another early event in PDAC development is mutation of the oncogene *KRAS2,* which is found altered in 20% of PanIN-1 lesions and this percentage increases with progression to invasive carcinoma. The tumor suppressor gene most commonly found mutated in PDAC is *CDKN2A*. This gene is found mutated in respectively 30%, 50% and 70% of PanIN-1, PanIN-2 and PanIN-3 lesions. Two additional important tumor suppressor genes in PDAC are *TP53* and *SMAD4*. In precursor lesions, mutations in these genes are mainly observed in PanIN-3 lesions in transition to invasive growth warranting *TP53/SMAD4* defects as a late event in PDAC development [16, 17]. In Figure 1 and Table 1, the most commonly observed specific genetic alterations in pre-invasive lesions of PDAC are mentioned, but many additional alterations exist. In order to understand pancreatic carcinogenesis, the whole

known spectrum of alterations has to be considered as well as the cellular interactions.

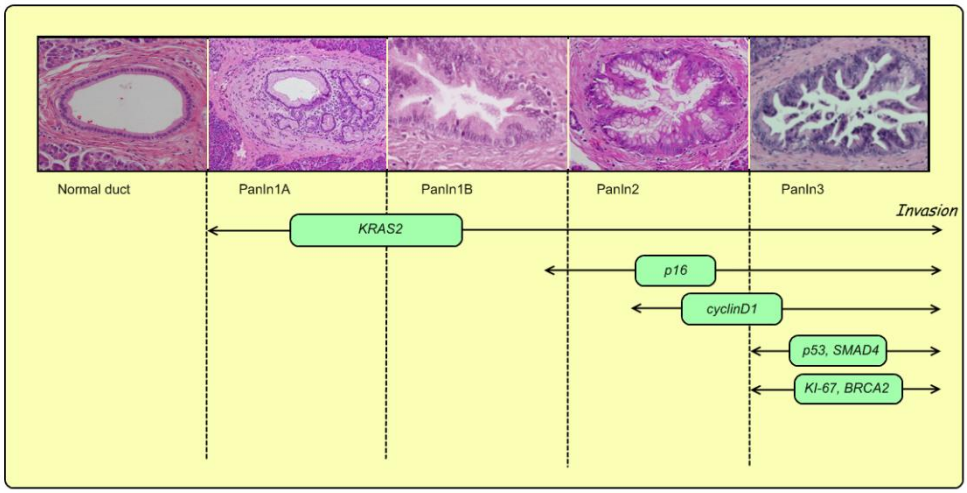


Figure 1 Progression model of pancreatic ductal adenocarcinoma from normal epithelium to invasively growing tumor. The progression is associated with the stepwise accumulation of specific genetic alterations depicted below the images.

Type	**Gene**	**Cellular function**	**Affected in PDAC**
Tumor suppressor genes	*CDKN2A/p16*	G1-S phase cell cycle inhibition	95%
	SMAD4	TGFβ	55%
	TP53	Cell cycle arrest	75%
Oncogenes	*KRAS2*	ERK-MAPkinase signaling	>90%
	CyclinD	Cell cycle progression	65%
	BRAF	ERK-MAPkinase signaling	5%
Genome maintenance genes	*MLH1/MSH2*	DNA damage (mismatch) repair	4%
	BRCA2	DNA damage repair	7-10%

Table 1 Most commonly affected genes in PDAC.

Molecular characteristics and regulatory pathways in PDAC

In 2008, Jones *et al.* used global genomic sequencing to identify the genetic alterations in pancreatic cancer cells. Over 21,000 genes were screened in 24

different PDAC samples. On average 63 relevant genetic alterations were found per sample, emphasizing the extreme complexity of this disease. These genetic alterations mostly affected 12, partially overlapping, signaling pathways that consequently contained abnormalities in the majority of cases [18, 19]. The identification of these pathways intelligently created a comprehensible view of pancreatic carcinogenesis without simplifying too much [19]. All previously known genetic alterations were included and put into the context of the pathways in which they function. Five of the pathways describe specific cellular functions; apoptosis, DNA damage repair, G1/S phase cell cycle progression, cell-cell adhesion and invasion (Figure 2). The other pathways are signaling cascades and can be divided into three groups: embryonic signaling pathways, the MAPkinase signaling pathways and TGF-β signaling. The molecular characteristics of PDAC are described within the context of these various specific pathways in the subsequent paragraphs. Table 2 gives an overview of the various affected pathways and their most commonly mutated genes in PDAC.

Regulatory pathway	**Affected genes**
Apoptosis	*TP53*
DNA damage repair	*TP53*
G1/S transition	*CDKN2A/p16, CyclinD*
Cell-cell adhesion	
Regulation of invasion	
Integrin signaling	
Homophilic cell adhesion	*CDH1*
Embryonic signaling	
Notch pathway	
Hedgehog pathway	
Wnt pathway	
MAPK signaling	
c-Jun N-terminal kinase	
ERK	*KRAS2*
TGF-β signaling	*SMAD4*

Table 2 The 12 commonly affected signaling pathways in PDAC accompanied by the most commonly affected genes from these pathways.

For the acquisition of an accumulation of genetic alterations by the neoplastic cells, genetic instability is a precondition [20]. Telomere shortening is considered as the initial neoplastic event that provides pancreatic epithelial cells the genetic instability that leads to the subsequently specific and generalized molecular alterations [21, 22].

Telomere shortening

Telomere shortening is encountered in virtually all pre-cursor lesions and invasive carcinomas [21, 23]. Telomeres are repeat sequences at the end of linear chromosomes that prevent fusion between the ends of these chromosomes. Pathologically short telomeres can result in ring and dicentric chromosomes that form so called anaphase bridges during mitosis. Breakage of these anaphase bridges generates highly recombinogenic free DNA ends, which in turn can result in chromosomal rearrangement. These cycles of chromosome bridging and breakage, called anaphase bridge-breakage-fusion cycles, repeat and thereby create the genetic instability that facilitates tumor development [24]. Telomerase, the gene that maintains telomere length, shows low expression during early pancreatic tumorigenesis before markedly increasing in the invasive tumor. The re-expression of telomerase probably restores genomic stability, enabling tumor progression by preventing further, possibly lethal, chromosomal damage [23, 25].

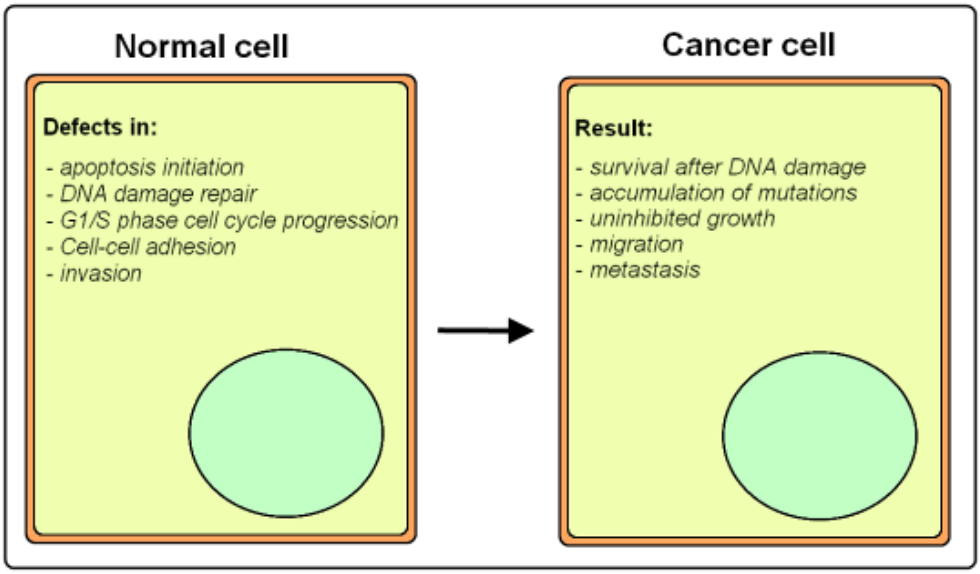


Figure 2 Cellular functions affected in pancreatic cancer.

Apoptosis

Apoptosis, or programmed cell death, plays an essential role in cancer development since resistance to apoptosis is a key factor in the survival of a cancer cell (Figure 3). Apoptosis is induced by executioner caspases upon activation by the apoptosome complex. This complex consists, among others, of cytochrome C, Caspase-9 and Apaf1 and is released from the mitochondria when pro-apoptotic signaling by Bak/Bax outweighs anti-apoptotic signaling by Bcl2, Bcl-

X(L) or Mcl-1. Inhibitors of apoptosis proteins (IAPs) can inhibit apoptosis at the end of the signaling cascade by direct inhibition of the executioner caspases. In PDAC, genes implicated in the apoptosis pathway were found altered in all tumors studied [19]. Also, previous reports document impaired apoptotic signaling in this disease. For example, a high fraction of apoptotic cells has been correlated with longer overall survival as well as absence of nodal involvement [26]. Moreover, most chemotherapeutics act through apoptosis induction whereby therapy resistance often is the result of defective apoptosis pathways.

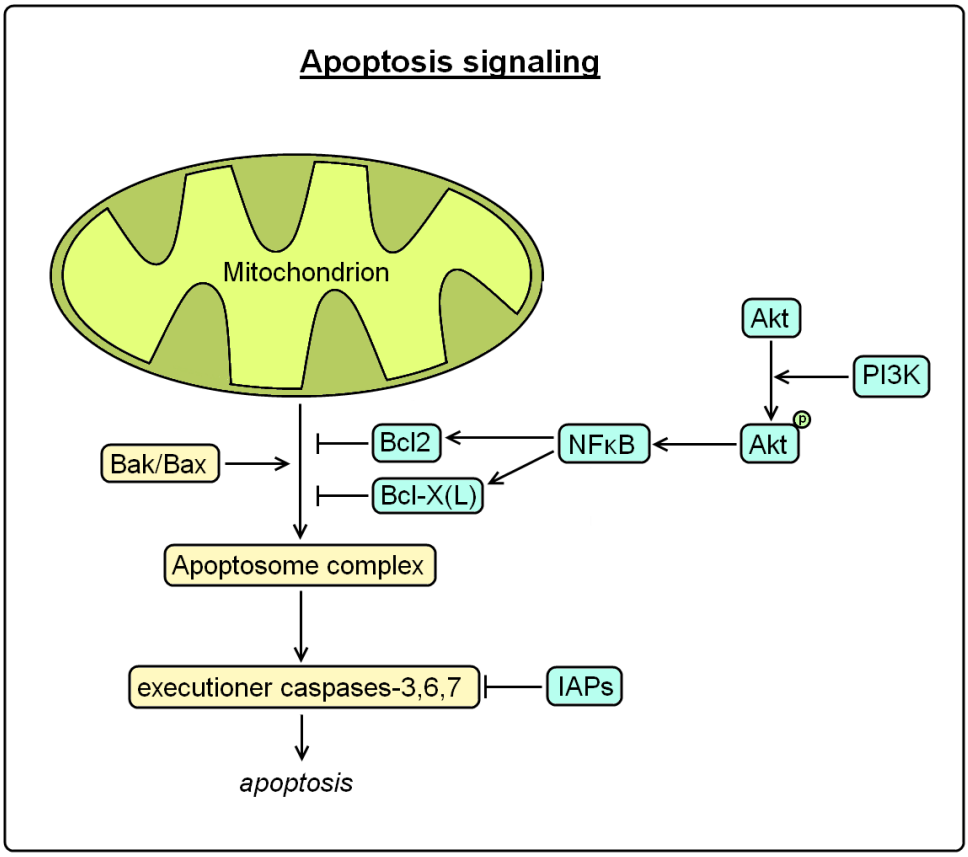


Figure 3 Apoptosis. Apoptosis is induced by executioner caspases upon activation by the apoptosome complex. This complex consists, among others, of cytochrome C, Caspase-9 and Apaf1 and is released from the mitochondria when pro-apoptotic signaling by Bak/Bax outweighs anti-apoptotic signaling by Bcl2/Bcl-X(L). PI3K activates Akt through phosphorylation which subsequently activates NF-kB. NF-kB stimulates anti-apoptotic signaling by Bcl2 and Bcl-X(L). Inhibitors of apoptosis proteins (IAPs) can inhibit apoptosis at the end of the signaling cascade by direct inhibition of the executioner caspases.

Anti-apoptotic genes *BCL-2*, *BCL-X(L)* and *MCL-1* are expressed in respectively 13%, 54% and 86% of PDAC samples as shown by immunohistochemistry, and repression of *BCL-2* and *MCL-1* was shown to enhance apoptosis in PDAC [27,

28]. The observed apoptotic effect was even more pronounced when treatment was combined with gemcitabine [29].

NF-κB is a transcription factor that regulates several different cellular mechanisms, of which most importantly apoptosis. NF-kB stimulates anti-apoptotic signaling by targeting *BCL-2 and BCL-X(L)* [30-32]. The NF-κB signaling pathway is activated by a variety of different mechanisms in PDAC amongst others oncogenic K-ras signaling [33]. Oncogenic K-ras signaling also activates phosphatidylinositol 3-kinase (PI3K), another important protein in apoptosis. PI3K activates Akt through phosphorylation which subsequently activates NF-kB.

The *AKT2* gene located on chromosome 19q is amplified in 10-20% of pancreatic cancers [34, 35] whereas PI3K/Akt signaling is activated in approximately 60% of PDACs [36]. PI3K signaling also involves mammalian target of rapamycin (mTOR), a downstream target of Akt. Activation of mTOR has been observed in approximately 75% of PDACs [37]. Therefore, inhibition of mTOR is an interesting target for therapy for which there are currently FDA approved inhibitors on the market. Although the exact role of the PI3K/Akt/mTOR pathway in pancreatic cancer remains to be elucidated, signaling of this pathway was shown to inhibit apoptosis and inhibition of the pathway increased cellular sensitivity to gemcitabine [38, 39].

DNA damage repair

DNA damage control genes are responsible for safeguarding the integrity of DNA as they code for proteins that repair any damage that occurs in the cell during its lifespan. An important DNA damage repair gene is *TP53*, a tumor suppressor gene located on chromosome 17p that is frequently disrupted in many different human malignancies. *TP53* expression is lost in 50-75% of PDACs [40, 41]. P53 is involved in the cellular response to genotoxic stress where it mediates cell cycle arrest and apoptosis upon DNA damage. Therefore, loss of *TP53* signaling results in a decrease in apoptosis and increases the opportunity for genetic alterations to accumulate in the cells.

Germline *BRCA2* gene mutations are responsible for ~10% of familial pancreatic cancer but mutations in this gene are also observed in approximately 7-10% of sporadic PDAC. The BRCA2 protein is involved in DNA damage repair, especially interstrand cross linking repair [42-44]. The BRCA2 gene will be further discussed in the paragraph on hereditary PDAC.

A third group of DNA damage repair genes involved in the development of PDAC is the mismatch repair family (MMR) of genes. The MMR proteins target base substitution mismatches and insertion–deletion mismatches that arise as a result of errors occurring during replication. Alterations in the mismatch repair genes *MLH1, MSH2, MSH6* and *PMS2* eventually lead to microsatellite instability (MSI) and this genetic instability makes the genome vulnerable for the accumulation of other,

more specific genetic alterations. Tumors of the pancreas with MSI are relatively rare compared to other malignancies of the digestive tract and are found in only 5% of pancreatic carcinomas. Pancreatic cancers with MSI have a distinct microscopic morphology that resembles their counterpart in the colon and are similarly called medullary type carcinomas [45-47]. Remarkably, microsatellite instable tumors have a significantly better prognosis compared to their microsatellite-stable counterparts [45, 46]. PDACs with MSI exhibit a higher anti-tumor reaction by T-lymphocytes and this could possibly be the reason for a better outcome [47].

G1/S phase cell-cycle progression

Cell cycle progression and regulation is affected in virtually all cancers as is the case for PDAC. Alterations in genes regulating G1/S-phase transition play an important role in facilitating the uncontrolled growth rate of cancer cells. The most commonly affected tumor suppressor gene in PDAC involved in G1/S phase transition is the *CDKN2A* gene [48, 49]. This gene is located on the short arm of chromosome 9 (9p21) and is known for its involvement in hereditary melanoma when mutated in the germline [50]. The gene is inactivated in >90% of all PDACs, either by homozygous deletion (40%) or an intragenic mutation combined with loss of heterogeneity of the remaining wild type allele (40%) [48, 51]. Promoter hypermethylation is the cause for loss of *CDKN2A* function in 15% of the cases [48]. P16, the protein product of CDKN2A inhibits phosphorylation of Rb-1, thereby preventing G1/S transition and acting as an inhibitory cell-cycle regulator [52]. Loss of p16 expression therefore leads to uncontrolled G1/S transition and unregulated cell division, which facilitates tumor progression [53].

Other genes involved in cell cycle progression that occasionally show alterations in PDAC are *FBXW7, CHD1* and *APC2*, although much less frequently than *CDKN2A* [19, 54].

Cell adhesion and invasion

In normal pancreatic tissue, cells are anchored to each other and their surroundings via multiple connections. A decrease in these interactions can allow cells to detach from their surrounding and migrate/metastasize. As such, cell to cell adhesion and interaction play an important role in carcinogenesis. The connection between epithelial cells is mostly mediated by the adherent junctions composed of E-cadherin and catenins. E-cadherin proteins interlock with each other in the extracellular space, while intracellulary the E-cadherin protein is bound to actin filaments through catenins. Reduced expression of E-cadherin, α- and ß-catenins was demonstrated in approximately 60%, 40% and 60% of pancreatic cancer samples, respectively [55, 56]. Reduced expression of E-cadherin is correlated with tumor dedifferentiation and correlates with tumor stage and lymph node involvement [55, 57]. Not only is the interaction between epithelial cells important in

the preservation of the integrity of the tissue, the interactions between the neoplastic cells and extracellular matrix also play an important role, especially in PDAC, because stromal tissue surrounds most tumor cells. Integrins comprise a large family of cell surface receptors and they act as a bridge between the extracellular matrix (ECM) and the cytoskeleton [58]. These integrins direct cell migration and play an important role in invasion. In addition to this, integrins also regulate cell proliferation and apoptosis. Integrin-ECM interactions are vital for cell survival but since apoptosis pathways are often affected in PDAC, loss of the interaction does not necessarily lead to apoptosis in cancer cells. Integrin signaling can activate the ERK, JNK MAPK pathway and the PI3K pathway, important pathways in pancreatic tumorigenesis. In approximately two-thirds of the PDAC cases, a defect in integrin signaling can be identified [19]. Different components of integrin signaling can be deregulated; for example, Integrin α6β1 expression has been correlated with metastatic behavior in pancreatic cancer cell lines [59]. Furthermore, Niu *et al.* investigated the role of αvβ6 integrin in PDAC and found that αvβ6 inhibition resulted in a significant reduction in cell proliferation and invasion. Apoptosis was induced and more remarkably, αvβ6 integrin knockdown increased gemcitabine sensitivity [60].

Another group of proteins involved in cell adhesion is the a-disintegrin and metalloproteinase (ADAM) protein family. ADAM proteins are cell surface proteins that activate MAPK pathways and integrin signaling through the release of growth factors. ADAM proteins have the ability to cleave ECM components and influence integrin/ECM interactions. ADAMs have only recently caught attention, thus not much is known about the specific role these proteins play in pancreatic carcinogenesis, though upregulation of different ADAM proteins has been reported in PDAC [61, 62]. Jones *et al.* found genetic alterations in various different ADAM proteins [19]. Because ADAM proteins influence many different substrates through autocrine and paracrine signaling they may comprise promising new targets for therapy development.

MAPK signaling pathways

There are three major mitogen activated phosphorylated kinases (MAPK): extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. All MAPK signaling pathways consist of the same basic kinase components. Stimulation of an upstream MAP2K kinase (MAP3K) by growth factors, stress, or other extracellular signals leads to phosphorylation of a MAPK kinase (MAP2K), culminating in the phosphorylation of a terminal MAPK (Figure 4). The most influential of the three MAPK pathways in PDAC is the ERK pathway. It consists of the Raf protein (MAP3K) that phosphorylates MEK (MAP2K), which in turn phosphorylates ERK (MAPK), the latter influencing transcription of different target genes. This signaling cascade results in the activation of multiple oncogenic

cellular functions. The most commonly mutated oncogene in PDAC is the *KRAS2* gene, of which the protein product Ras is an upstream activator of ERK signaling. *KRAS2* is located on chromosome 12p and the protein has an intrinsic GTP-ase activity. In PDAC the gene is virtually always activated by a point mutation in codon 12, the GTP binding domain, leading to a constitutively active Ras protein [63, 64]. Therefore, it can be considered a molecular switch that in this fashion remains in the 'on' position firing its oncogenic stimuli.

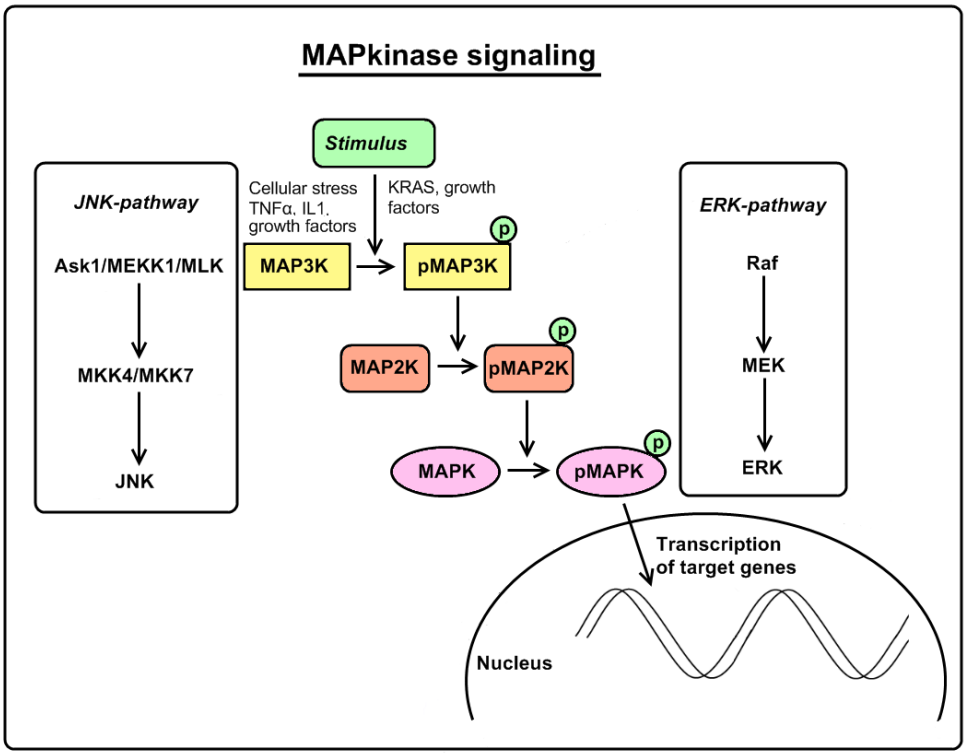


Figure 4 MAPkinase signalling. Mitogen activated phosphorylated kinase signaling occurs through a common pathway. A cellular stimulus results in phosphorylation of MAP3K, which in turn phosphorylates MAP2K. MAP2K subsequently phosphorylates MAPK, resulting in altered transcription of the MAPK target genes. The different components of the two MAPK signaling pathways often affected in PDAC, the ERK pathway and the JNK pathway, are depicted in the boxes on each side of the signaling cascade.

As said before, *KRAS2* gene mutations are an early phenomenon in the development of PDAC and the *KRAS2* gene is mutated in ~95% of PDACs. Interestingly, the few tumors that contain wild-type *KRAS2* often have a mutation in the *BRAF* gene, an oncogene located on chromosome 7q. The *BRAF* gene is mutated in approximately 5% of the PDACs and Raf, the protein product of *BRAF*, is a downstream target in the Ras signaling pathway. This explains the mutually exclusive nature of *KRAS2* and *BRAF* mutations in PDAC [54]. The high frequency and early nature of *KRAS2* mutations suggests an initiating role in PDAC

development as confirmed in several studies on genetically engineered mice [65, 66]. Besides the effects Ras has on the ERK-pathway, Ras also influences multiple other genes among which NF-kB and PI3K/Akt as discussed above.
The second MAPK pathway often affected in PDAC is the JNK pathway. In the whole genome sequence analysis study by Jones *et al*. mentioned earlier, in all but one of the sequenced samples a genetic alteration in the JNK pathway was identified [19]. In this signaling cascade the MAP3Ks, Ask1, MEKK1 and MLK, phosphorylate the MAP2Ks, MKK4 and MKK7 which in turn phosphorylate JNK. The JNK pathway becomes activated upon cellular stress but more importantly, the pathway is activated by pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) or interleukin 1 (IL1) [67]. *MKK4* expression is lost in approximately 4-15% of PDACs [68, 69]. Remarkably, the JNK pathway has both tumor suppressor and oncogenic functions that have to be further investigated. Also it should be noted that the Kras and the JNK pathways interact; phosphorylation of JNK is partly responsible for induction of angiogenesis through Kras [70]. Recent studies have also connected MKK4 and its downstream targets (JNK and p38) to the TGF-β pathway.

TGF-β pathway
The transforming growth factor β (TGF-β) pathway has been linked to PDAC for many years. TGF-β signaling is involved in a wide range of cellular processes [71]: It is one of the most potent cell proliferation inhibitors and has many other cellular responsibilities including differentiation, apoptosis and angiogenesis [72]. Binding of a TGF-β family ligand to the TGF-βII receptor leads to phosphorylation of the TGF-βI receptor and thereby activation of the TGF-β receptor substrates capable of signal transduction, i.e. the Smad family proteins. Eight different *SMAD* genes have been described. Once activated, the receptor subsequently phosphorylates a regulatory Smad (Smad1-3, 5, 8), allowing this protein to associate with Smad4. The latter aids the regulatory Smad complex in its transfer to the nucleus where subsequent transcription of the target genes is induced. Inhibitory Smads regulate Smad-signaling through inhibition of the TGF-β receptor phosphorylation. Thus far, Smad7 is the only characterized inhibitory Smad (Figure 5) [71].

TGF-β influences cellular proliferation through inhibition of G1/S-transition. This is accomplished though expression of cyclin kinase inhibitors such as p15, p21 and p27 [73]. Also, TGF-β signaling represses c-Myc expression, an ubiquitous promoter of cell cycle progression. Jones *et al*. found altered TGF-β pathway expression in all their PDAC samples [19]. The most commonly affected protein in the TGF-β pathway is Smad4. Smad4 is inactivated in ~55% of PDACs [74, 75]. Patients with preserved Smad4 signaling have a significantly longer survival than patients with Smad4 loss [75-77]. Also, Iacobuzio-Donahue *et al.* found a

significantly higher percentage of Smad4 loss in patients who had died from PDAC with widespread metastatic disease compared to patients who died of locally advanced tumors [77]. Loss of Smad4 expression is not only a prognosticator but it can also serve as a diagnostic biomarker since sensitive and specific antibodies are available that can be used to characterize Smad4 protein expression by immunohistochemistry (Figure 6) [76]. Other proteins in the TGF-β pathway that are occasionally found altered in PDAC are the TGF-βRII (4%) and TGF-βRI (1%) [78]. Apart from binding to the TGF-βR, TGF-β ligands can also activate other signaling pathways including the MAPK pathways ERK and JNK [79-81]. This depicts the fact that although TGF-β signaling has a tumor suppressive function in the normal epithelium, it can promote tumor progression in late disease stages. Further research has to be conducted to determine the true potential of this pathway for the development of targeting agents.

Embryonic pathways

Not surprisingly, since embryogenesis shares many characteristics with carcinogenesis, different embryonic pathways are involved in tumor development. There are three embryonic pathways involved in pancreatic carcinogenesis: Notch, Hedgehog and Wnt (Figure 5).

The Notch pathway plays an important role in pancreatic organogenesis, but after formation of the pancreas, signaling is largely restricted to a putative progenitor population known as centroacinar cells [82-85]. Several studies have shown upregulation of Notch pathway activity in PDAC and inhibition of this pathway resulted in decreased tumor proliferation and increased apoptosis [85-88]. Somatic point mutations in one of the four Notch-receptor genes do not seem to be the driving force behind altered Notch signaling in pancreatic cancer [88]. Still, 100% of the PDAC samples in a genome wide sequencing study revealed alterations in the Notch pathway [19]. Notch signaling interacts with many other oncogenic pathways including the Hedgehog pathway, KRAS signaling and the NF-κB pathway.

The second embryonic pathway often affected in PDAC is the Hedgehog (Hh) pathway. This signaling cascade plays an important role in the organogenesis of the gastro-intestinal tract. Surprisingly, Hh signaling is absent in the developing pancreas [89, 90] but the pathway is activated in 70% of PDACs [91]. Some of the Hh signaling targets are components of other signaling pathways involved in PDAC such as Wnt proteins, TGF-β and CyclinD [92-94].

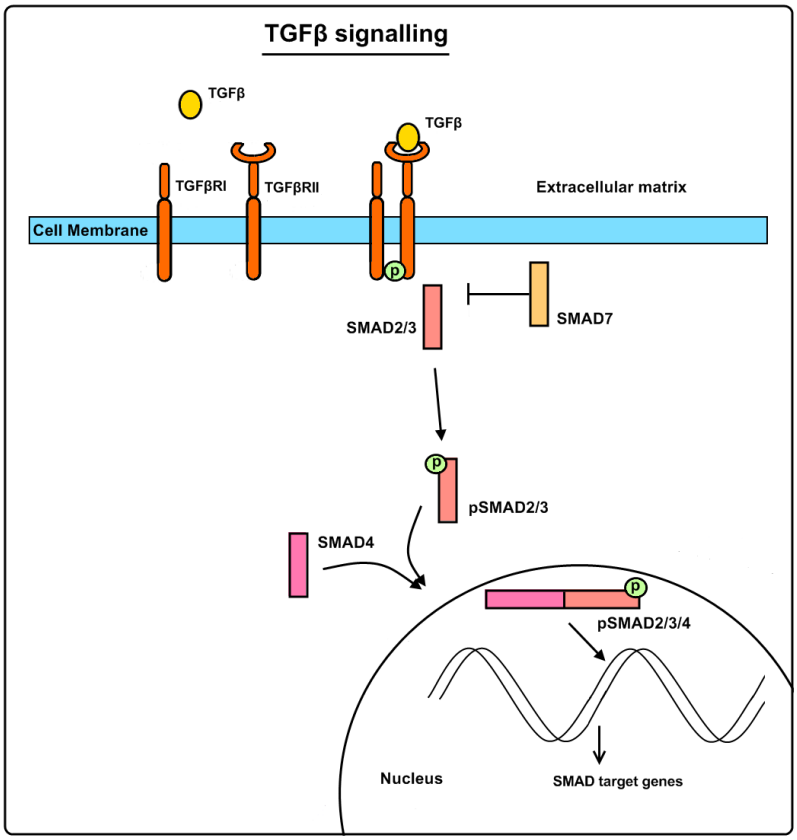


Figure 5 The TGF-β signaling pathway. The TGF-β signaling pathway is activated by binding of TGF-β to a type II receptor, which facilitates the recruitment and phosphorylation of the type I receptor. This pTGF-βRI activates either SMAD2 or SMAD3 by phosphorylation. The phosphorylated SMAD2/3 forms a complex with SMAD4 and transports to the nucleus where it influences SMAD target gene transcription. SMAD7 can inhibit TGF-β signaling through inhibition of TGF-βRI phosphorylation.

Although it has long been common knowledge that Hh signaling is active in PDAC, its exact role in tumorigenesis is unclear. It seems that neoplastic epithelial cells do not have the ability to react to Hh signaling. Instead, Hh ligands are expressed in the epithelial cells and it has been suggested that these effect the stromal compartment of a tumor through paracrine signaling. In one particular study the strong desmoplastic reaction characteristic for PDAC, was shown to be further enhanced when Hh signaling was activated [95]. In addition, inhibition of the Hh pathway decreased the total volume of orthotopically implanted tumors by inhibiting the stromal component in mice [95]. Another study showed that treatment with a Hh pathway inhibitor produced a clear decrease in tumor growth primarily through a decrease in number of stromal cells [96]. Similarly, disruption of Hh signaling in a transgenic mouse model increased response to chemotherapy [97]. This improved response was due to a diminished desmoplastic reaction and better accessibility of

the tumor cells for the chemotherapeutic agent. In short, there seems to be an important role for Hh signaling in the stromal component of PDAC. Moreover, since the desmoplastic reaction has been related to resistance to therapy it warrants further investigation of Hh and its role in the development of PDAC. Clinical trials with inhibitors of hedgehog signaling are in progress.

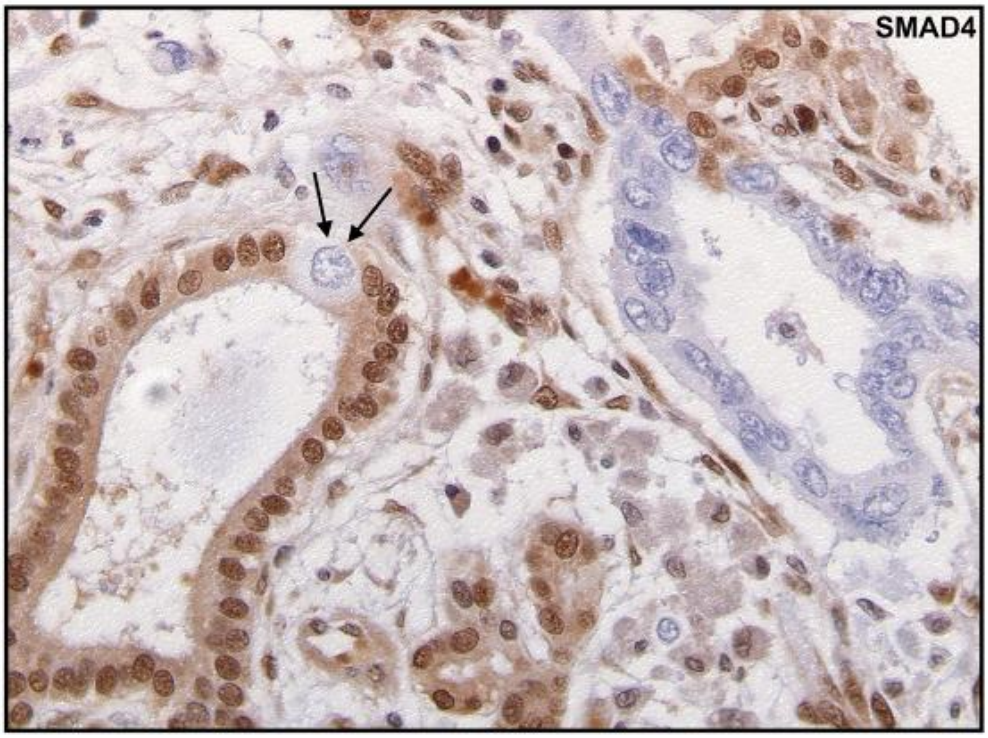


Figure 5 SMAD4 immunohistochemistry. Loss of *SMAD4* expression is clearly depicted in the PDAC cells. Arrows: single cell with clear histological changes exhibiting SMAD4 loss, surrounded by *SMAD4* wild type cells.

The third embryonic pathway, Wnt, shows increased activity in approximately 30-65% of PDACs and an increase in Wnt-target expression correlates with poorer differentiation and poor prognosis [98-100]. Active Wnt expression results in the transcription of different target genes including *CyclinD*, matrix metalloproteinase 7 (*MMP7*) and *c-MYC*. CyclinD is overexpressed in 65% of PDACs and stimulates G1/S transition. Expression of this protein is associated with poor prognosis [101]. MMP7, a member of the matrix metalloproteinase family, degrades extracellular matrix proteins and MMP7 expression is implicated in metastases. Overexpression of this protein is found in practically all PDACs [102].

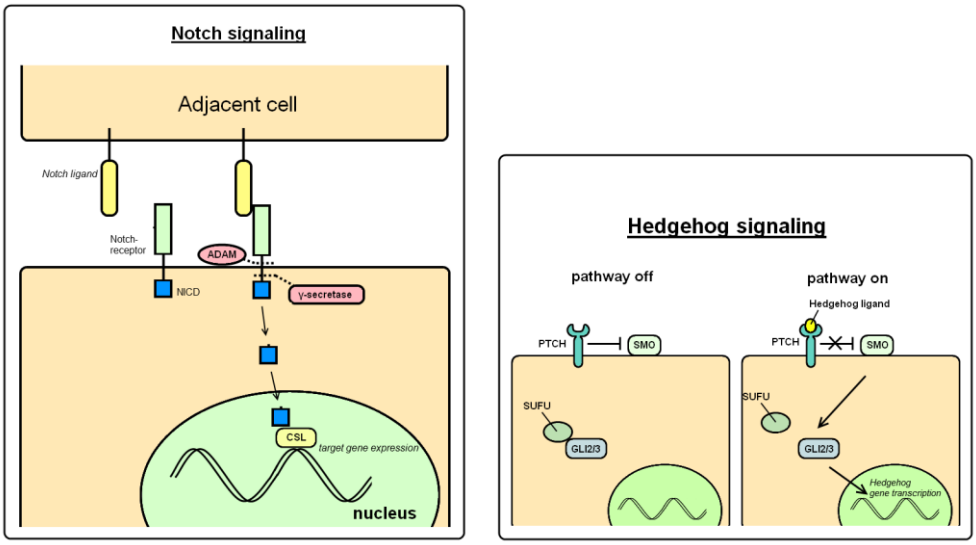


Figure 7 ***Left*** Notch signaling. Upon Notch ligand binding, ADAM performs the first cleavage quickly followed by the second cleavage performed by γ-secretase. This second cleavage releases the Notch intracellular domain (NICD) into the cellular lumen. NICD transports to the nucleus where it interacts with a transcription domain resulting in transcription of the Notch target genes.
Right Hedgehog signaling. **In** normal pancreatic cells Hedgehog signaling is repressed; the patched (PTCH) receptor represses the smoothened (SMO) receptor. Intracellularly, the hedgehog inhibitor suppressor of fused (SUFU) binds the GLI family zink finger transcription factors GLI2/3 thereby inducing proteasomal cleavage resulting in repressor forms of GLI2/3. When one of the Hedgehog ligands attaches to Patched this abrogates the inhibition on Smoothened. Smoothened inhibits proteasomal cleavage of GLI2/3 and thereby facilitates transcriptional activity.

C-Myc is a transcription factor that regulates thousands of genes involving a large spectrum of cellular functions including cell proliferation, differentiation, death, and tissue re-organization. Amplification of *CMYC* is identified in 20-50% of all PDACs [103]. The Wnt signaling cascade can be activated through interactions with the Hh, NF-kB, TGF-β and Notch pathways [104-106].

Genetic susceptibility

Approximately 5-10% of patients with pancreatic cancer have a positive family history for the disease. Having a first-degree relative with PDAC doubles the chance of developing pancreatic cancer compared to individuals without such a history, and the risk increases with increasing number of affected relatives suggesting a hereditary component. Some of these PDACs arise in the setting of a known familial cancer syndrome, however in most instances the genetic basis for the familial aggregation is not known [107].
To date, at least 5 hereditary disorders that significantly increase the chance of pancreatic cancer development have been described. These include familial

atypical multiple melanoma and mole (FAMMM) syndrome, Peutz-Jeghers syndrome, hereditary pancreatitis, familial breast cancer and other syndromes related to alterations in Fanconi anemia genes, and the Lynch syndrome (Table 3).
The FAMMM syndrome is caused by germline mutations in the *CDKN2A* gene. As stated before, this gene is often somatically mutated in sporadic pancreatic cancer. Patients suffering from this syndrome have a 20-34 fold risk of developing PDAC [108]. This risk is especially high when the mutation is a specific 19-base-pair deletion in *CDKN2A*; the p16-Leiden deletion [109].

Syndrome	**Affected gene(s)**	**RR of PDAC**
Familial atypical multiple melanoma and mole syndrome	*CDKN2A*	20-34
Peutz-Jeghers syndrome	*LKB1*	>100
Hereditary pancreatitis	*PRSS1/SPINK1*	~90
Familial breast cancer	*BRCA1/2*	3-10
Lynch syndrome	*mismatch repair genes*	unknown

Table 3 Hereditary syndromes associated with an increased risk of PDAC development.

Peutz-Jeghers syndrome is caused by mutations in the *STK11/LKB1* gene, a serine threonine kinase involved in a large number of cellular functions, from control of cell polarity to metabolism. Patients suffering from this syndrome have a 132-fold increased risk of developing PDAC with a 30-60% lifetime risk of PDAC at age 70 [110-112].

Patients with hereditary pancreatitis develop recurrent episodes of pancreatitis, starting at a young age. The syndrome is most commonly caused by mutations in the cationic trypsinogen gene *PRSS1* [113]. Another gene that is occasionally found altered in patients with hereditary pancreatitis is the serine peptidase inhibitor *SPINK1* [114]. Carriers of either of these mutations have a highly increased risk of developing pancreatic cancer with a lifetime risk of 25-40% at age 60.
The two *BRCA* genes are best known for their role in familial breast and ovarian cancer but *BRCA2* also plays a role in pancreatic cancer development. Carriers of germline *BRCA2* gene mutations have a 3-10 fold increased risk of developing PDAC. A specific interest goes to the Ashkenazi Jewish population as approximately 1% of Ashkenazi Jews are carriers of a founder *BRCA2* mutation, 6174delT [115].
Fanconi anemia is a hereditary cancer susceptibility disorder, with occurrence of multiple haematological malignancies. The Brca2 protein interacts with different Fanconi anemia pathway components, and the corresponding encoding genes, especially *FANCC* and *FANCG,* have also been reported to increase the chance of

PDAC development when mutated. Recently, *PALB2*, yet another FANC gene was reported to be responsible for ~3% of the cases of familial pancreatic cancer [116, 117]. *PALB2* encodes a protein that enables the localization and binding of Brca2 to sites of double strand DNA breaks.
Lynch syndrome is caused by germline mutations in a number of DNA mismatch repair genes. Patients suffering from the syndrome have a slightly increased chance of developing pancreatic cancer although there is still some debate about the exact role in PDAC development [118].

Identification of germline mutations in the previously discussed genes is of great importance, not only for screening purposes but also because they could potentially hold therapeutic consequences. Furthermore, no genetic basis for cancer susceptibility is identified in most cases of families exhibiting high numbers of PDAC affected individuals. More research on genetic susceptibility for PDAC will have to be conducted to explain the genetic basis for disease development.

Treatment of PDAC

Adjuvant therapy after resection of the tumor consisting of gemcitabine has been the treatment of choice since 1997 when it was shown to improve both disease free and overall survival [5, 6]. Several studies examining the effect of adding other therapeutic agents to gemcitabine have been conducted over the past years with disappointing results [119, 120]. Only the addition of erlotinib showed slight improvement of overall survival [121]. A recently published report confirmed the earlier observed limited beneficial effect of adding erlotinib, however, the authors concluded that this was no justification for a phase III trail [122]. Reports comparing single-agent gemcitabine to adjuvant chemoradiation therapy have been inconclusive. Chemo-radiation therapy has been implicated in the USA since the Gastro-Intestinal Tumor Study Group trial was published which showed longer overall survival in patients treated with adjuvant chemoradiation [123, 124]. In Europe however, a similar study failed to find a significant survival advantage for the group receiving additional radiotherapy thus chemoradiation therapy did not become the standard treatment [125]. Although the most recent reports on this subject suggest a significant advantage for the addition of radiotherapy, there is still controversy about this subject and more research needs to be done before radiation therapy can be included as standard first-line of treatment for PDAC in Europe [123]. It has been suggested that neoadjuvant treatment with chemotherapy, radiation therapy or chemoradiation therapy could downstage borderline resectable tumors. Several recent studies have shown promising results for treating borderline resectable tumors with chemoradiation, enabling resection and approaching similar survival rates as truly resectable tumors [126-129]. This is

still under investigation and future studies will have to be conducted to justify the use of neoadjuvant treatment.
Approximately 80% of patients present with locally or systemically advanced disease making resection redundant. For these patients only palliative treatment options remain. Single agent gemcitabine is currently recommended as standard first-line chemotherapy for patients with advanced disease [5].

Since the arrival of whole genome sequencing, it has become possible to identify all the genomic alterations that lead to the development of pancreatic cancer. The next logical step is to translate this knowledge into better treatment options. Until recently, no targeted agents were found to improve outcome in the clinical setting although many studies have shown promise in the *in vitro* setting. In the past year, a group used mutation analysis to guide their treatment strategy for the first time [130]. Earlier studies had shown that PDAC cell lines harboring mutations in the above mentioned *BRCA2* gene, but also other genes related to the Fanconi Anemia syndrome (*FANCC, FANCG*), responded better to treatment with interstrand cross linking (ICL) agents than *FANC/BRCA* wild-type tumors [131]. The *FANC/BRCA* pathway is involved in the repair of double stranded DNA-breaks. As ICL-forming agents induce this type of DNA damage, susceptibility of *FANC/BRCA* mutated tumors to the ICL-forming agents seemed reasonable. Showalter *et al.* performed mutation analysis for *BRCA2* and one patient harboring a mutation in this gene was treated with cisplatin, an ICL agent, in addition to gemcitabine showing favorable results (the patient is still alive after 32 months). In theory, PDACs carrying *PALB2* mutations should be sensitive to the same targeted therapeutic as *PALB2* is a binding partner of *BRCA2*. Trials justifying use of ICL agents in *PALB2* mutated PDAC still have to be conducted.
We have recently seen a Peutz-Jeghers syndrome patient with pancreatic cancer whose tumor showed complete loss of *LKB1*, an inhibitor of mTOR. This patient responded to treatment with everolimus, one of the known mTOR inhibitors used in clinical setting. Specifically, the tumor diminished in size by more than 50% within 6 months, but became resistant thereafter [132].
Inhibition of Kras signaling with farnesyl transferase inhibitors used in the past did not have a beneficial effect (reviewed by [133]). In 2010, a new therapeutic agent was identified targeting the Kras pathway. Protein Kinase C iota (PKC iota) was shown to drive transformed growth in pancreatic cancer cell lines via inhibition of oncogenic Kras activity and inhibition of PKC iota resulted in a significant reduction of metastases and invasion in preclinical models [134]. Further research has to be done to map the effectiveness of inhibiting PKC iota *in vivo*.
MTAP, a gene located near *CDKN2A*, is co-deleted with the *CDKN2A* gene in 30 % of the pancreatic cancers. *MTAP* might be a possible therapeutic target as

approaches to selectively target cells with *MTAP* defects have already been developed [135, 136]. However, these have not been tested in a clinical setting yet. It seems logical that over the next few years multiple small steps, hopefully adding up to significant progress, will be taken on the road to targeted treatment of PDAC.

Conclusion

The aim of this review was to emphasize the complexity of tumorigenesis in pancreatic ductal adenocarcinoma and to provide an introductory overview of the pathways affected in PDAC. As the knowledge on tumorigenesis of PDAC expands rapidly, so do the possibilities to design more effective treatment. The arrival of genome sequencing has offered the opportunity to establish an overview of the genetic alterations that lead to tumor development and this could subsequently play an important role in our search for new therapeutic targets. The complexity of the genetics accompanying PDAC indicates that it is impossible to design a treatment that fits all. From this can be deducted that personalized treatment based on tumor genotyping will probably be most effective and feasible. The use of ICL agents for tumors harboring *BRCA2* mutations is the first step in that direction.
Based on the data presented in this review, it seems advisable to shift the focus of research from most commonly affected genes to the most commonly affected pathways as some important yet rare alterations could be missed. The interactions all these pathways undergo are extensive and complex as mentioned earlier. When considering personalizing treatment, designing workable and quick tumor characterizing assays and targeting pathways rather than individual genes seems to hold the future of cancer therapy in PDAC.

Reference list

1. Jemal A, Siegel R, Ward S, et al., *Cancer statistics, 2009*. CA Cancer J Clin, 2009. 59(4): p. 225-49.
2. Yeo TP, Hruban RH, Leach SD, et al., *Pancreatic cancer.* Curr Probl Cancer, 2002. 26(4): p. 176-275.
3. Riall TS, Cameron JL, Lillemoe KD, et al., *Pancreaticoduodenectomy with or without distal gastrectomy and extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma--part 3: update on 5-year survival.* J Gastrointest Surg, 2005. 9(9): p. 1191-204
4. Maitra A, Kern SE and Hruban RH, *Molecular pathogenesis of pancreatic cancer.* Best Pract Res Clin Gastroenterol, 2006. 20(2): p. 211-26.
5. Burris HA, Moore MJ, Andersen MR, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* J Clin Oncol, 1997. 15(6): p. 2403-13.
6. Oettle H, Post S, Neuhaus P, et al., *Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial.* Jama, 2007. 297(3): p. 267-77.
7. Abbott DE, Baker MS and Talamonti MS, *Neoadjuvant therapy for pancreatic cancer: a current review.* J Surg Oncol, 2010. 101(4): p. 315-20.
8. Bramhall SR, Schulz J, Nemunaitis J, et al., *A double-blind placebo-controlled, randomised study comparing gemcitabine and marimastat with gemcitabine and placebo as first line therapy in patients with advanced pancreatic cancer.* Br J Cancer, 2002. 87(2): p. 161-7.
9. Van Cutsem E, van de Velde H, Karasek P, et al., *Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer.* J Clin Oncol, 2004. 22(8): p. 1430-8.
10. Van Cutsem E, Vervenne WL, Bennouna J, et al., *Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer.* J Clin Oncol, 2009. 27(13): p. 2231-7.
11. Singh M and Maitra A, *Precursor lesions of pancreatic cancer: molecular pathology and clinical implications.* Pancreatology, 2007. 7(1): p. 9-19.
12. Campbell PJ, Yachida S, Mudie LJ, et al., *The patterns and dynamics of genomic instability in metastatic pancreatic cancer.* Nature, 2010. 467(7319): p. 1109-13.
13. Hruban RH, Takaori K, Klimstra DS, et al., *An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms.* Am J Surg Pathol, 2004. 28(8): p. 977-87.
14. Kargozaran H, Vu V, Ray P, et al., *Invasive IPMN and MCN: same organ, different outcomes?* Ann Surg Oncol, 2010.
15. Biankin AV, Kench JG, Biankin SA, et al*., Pancreatic intraepithelial neoplasia in association with intraductal papillary mucinous neoplasms of the pancreas: implications for disease progression and recurrence.* Am J Surg Pathol, 2004. 28(9): p. 1184-92.
16. Koorstra JB, Hong SM, Shi C, et al., *Widespread activation of the DNA damage response in human pancreatic intraepithelial neoplasia.* Mod Pathol, 2009. 22(11): p. 1439-45.
17. Wilentz RE, Goggins M, Redston M, et al., *Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression.* Cancer Res, 2000. 60(7): p. 2002-6.
18. Carter H, Samayoa J, Hruban RH, et al., *Prioritization of driver mutations in pancreatic cancer using cancer-specific high-throughput annotation of somatic mutations (CHASM).* Cancer Biol Ther, 2010. 10(6).

19. Jones S, Zhang X, Parsons DW, et al., *Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science, 2008. 321(5897): p. 1801-6.
20. Lengauer C, Kinzler KW, Vogelstein B, et al., *Genetic instabilities in human cancers.* Nature, 1998. 396(6712): p. 643-9.
21. van Heek NT, Meeker AK, Kern SE, et al., *Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia.* Am J Pathol, 2002. 161(5): p. 1541-7.
22. Artandi SE and DePinho RA, *Telomeres and telomerase in cancer.* Carcinogenesis, 2010. 31(1): p. 9-18.
23. Gisselsson D, Jonson T, Petersen A, et al., *Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors.* Proc Natl Acad Sci U S A, 2001. 98(22): p. 12683-8.
24. Artandi SE, Chang S, Lee SL, et al., *Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice.* Nature, 2000. 406(6796): p. 641-5.
25. Hackett JA and Greider CW, *Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis.* Oncogene, 2002. 21(4): p. 619-26.
26. Magistrelli P, Coppola R, Tonini G, et al., *Apoptotic index or a combination of Bax/Bcl-2 expression correlate with survival after resection of pancreatic adenocarcinoma*. J Cell Biochem, 2006. 97(1): p. 98-108.
27. Virkajarvi N, Paakko P and Soini Y, *Apoptotic index and apoptosis influencing proteins bcl-2, mcl-1, bax and caspases 3, 6 and 8 in pancreatic carcinoma.* Histopathology, 1998. 33(5): p. 432-9.
28. Evans JD, Cornford PA, Dodson A, et al., *Detailed tissue expression of bcl-2, bax, bak and bcl-x in the normal human pancreas and in chronic pancreatitis, ampullary and pancreatic ductal adenocarcinomas.* Pancreatology, 2001. 1(3): p. 254-62.
29. Banerjee S, Choi M, Aboukameel A, et al., *Preclinical studies of apogossypolone, a novel pan inhibitor of bcl-2 and mcl-1, synergistically potentiates cytotoxic effect of gemcitabine in pancreatic cancer cells*. Pancreas, 2010. 39(3): p. 323-31.
30. Wang CY, Mayo MW, Korneluk RG, et al., *NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation.* Science, 1998. 281(5383): p. 1680-3.
31. Wang CY, Guttridge DC, Mayo MW, et al., *NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis.* Mol Cell Biol, 1999. 19(9): p. 5923-9.
32. Kreuz S, Siegmund D, Scheurich P, et al., *NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling.* Mol Cell Biol, 2001. 21(12): p. 3964-73.
33. Holcomb B, Yip-Schneider M and Schmidt CM, *The role of nuclear factor kappaB in pancreatic cancer and the clinical applications of targeted therapy.* Pancreas, 2008. 36(3): p. 225-35.
34. Cheng JQ, Ruggeri B, Klein WM, et al., *Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA.* Proc Natl Acad Sci U S A, 1996. 93(8): p. 3636-41.
35. Ruggeri BA, Huang L, Wood M, et al., *Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas.* Mol Carcinog, 1998. 21(2): p. 81-6.
36. Schlieman MG, Fahy BN, Ramsamooj R, et al., *Incidence, mechanism and prognostic value of activated AKT in pancreas cancer.* Br J Cancer, 2003. 89(11): p. 2110-5.
37. Bellizzi AM, Bloomston M, Zhou XP et al., *The mTORpPathway is frequently activated in pancreatic ductal adenocarcinoma and chronic pancreatitis.* Appl Immunohistochem Mol Morphol, 2010 18(5):442-7.

38. Arlt A, Gehrz A, Muerkoster S, et al., *Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death.* Oncogene, 2003. 22(21): p. 3243-51.
39. Roy SK, Srivastava RK, and Shankar S, *Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer.* J Mol Signal, 2010. 19;5:10.
40. Redston MS, Caldas C, Seymour AB, et al*., p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions.* Cancer Res, 1994. 54(11): p. 3025-33.
41. Barton CM, Staddon SL, Hughes CM, et al., *Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer.* Br J Cancer, 1991. 64(6): p. 1076-82.
42. Schutte M, Hruban RH, Geradts J. et al*., Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region.* Proc Natl Acad Sci U S A, 1995. 92(13): p. 5950-4.
43. van der Heijden MS, Brody JR, Dezentje DA, et al., *Functional defects in the fanconi anemia pathway in pancreatic cancer cells.* Am J Pathol, 2004. 165(2): p. 651-7.
44. Goggins M, Schutte M, Lu J, et al., *Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas.* Cancer Res, 1996. 56(23): p. 5360-4.
45. Nakata B, Wang YQ, Yashiro M, et al., *Prognostic value of microsatellite instability in esectable pancreatic cancer.* Clin Cancer Res, 2002. 8(8): p. 2536-40.
46. Goggins M, Offerhaus GJA, Hilgers W et al., *Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+.* Am J Pathol, 1998. 152(6): p. 1501-7.
47. Yamamoto H, Itoh F, Nakamura H, et al., *Genetic and clinical features of human pancreatic ductal adenocarcinomas with widespread microsatellite instability. Cancer Res, 2001. 61(7): p. 3139-44.*
48. Schutte M, Hruban RH, Geradts J, et al., *Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas.* Cancer Res, 1997. 57(15): p. 3126-30.
49. Wilentz RE, Geradts J, Maynard R, et al., *Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression.* Cancer Res, 1998. 58(20): p. 4740-4.
50. Begg CB, Orlow I, Hummer AJ, et al*., Lifetime risk of melanoma in CDKN2A mutation carriers in a population-based sample.* J Natl Cancer Inst, 2005. 97(20): p. 1507-15.
51. Caldas C, Hahn SA, da Costa LT, et al*., Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma.* Nat Genet, 1994. 8(1): p. 27-32.
52. Serrano M, Hannon GJ and Beach D, *A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4.* Nature, 1993. 366(6456): p. 704-7.
53. Maitra, A. and Hruban R.H., *Pancreatic cancer.* Annu Rev Pathol, 2008. 3: p. 157-88.
54. Calhoun ES, Jones JB, Ashfaq R, et al., *BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) mutations in distinct subsets of pancreatic cancer: potential therapeutic targets.* Am J Pathol, 2003. 163(4): p. 1255-60.
55. Joo YE, Rew JS, Park CS, et al., *Expression of E-cadherin, alpha- and beta-catenins in patients with pancreatic adenocarcinoma.* Pancreatology, 2002. 2(2): p. 129-37.

56. Li YJ and Ji XR, *Relationship between expression of E-cadherin-catenin complex and clinicopathologic characteristics of pancreatic cancer.* World J Gastroenterol, 2003. 9(2): p. 368-72.
57. Winter JM, Ting AH, Vilardellet F, et al., *Absence of E-cadherin expression distinguishes noncohesive from cohesive pancreatic cancer*. Clin Cancer Res, 2008. 14(2): p. 412-8.
58. Giancotti FG and Ruoslahti E, *Integrin signaling*. Science, 1999. 285(5430): p. 1028-32.
59. Sawai H, Takeyama H, Yamamoto M, et al., *Enhancement of integrins by interleukin-1alpha, and their relationship with metastatic and invasive behavior of human pancreatic ductal adenocarcinoma cells.* J Surg Oncol, 2003. 82(1): p. 51-6.
60. Niu W, Liu X, Zhang Z, et al., *Effects of alphavbeta6 gene silencing by RNA interference in PANC-1 pancreatic carcinoma cells.* Anticancer Res. 2011 30(1): p. 135-42.
61. Yamada D, Ohuchida K, Mizumoto K, et al*., Increased expression of ADAM 9 and ADAM 15 mRNA in pancreatic cancer.* Anticancer Res, 2007. 27(2): p. 793-9.
62. Grutzmann R, Luttges J, Sipos B, et al., *ADAM9 expression in pancreatic cancer is associated with tumour type and is a prognostic factor in ductal adenocarcinoma.* Br J Cancer, 2004. 90(5): p. 1053-8.
63. Hruban RH, van Mansfeld AD, Offerhaus GJ, et al*., K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization.* Am J Pathol, 1993. 143(2): p. 545-54.
64. Almoguera C, Shibata D, Forrester K, et al., *Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes.* Cell, 1988. 53(4): p. 549-54.
65. Aguirre AJ, Bardeesy N, Sinha M,et al., *Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma.* Genes Dev, 2003. 17(24): p. 3112-26.
66. Hingorani SR, Petricoin EF, Maitra A, et al., *Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse.* Cancer Cell, 2003. 4(6): p. 437-50.
67. Davis RJ, *Signal transduction by the JNK group of MAP kinases.* Cell, 2000. 103(2): p. 239-52.
68. Xin W, Yun KJ, Ricci F, et al., *MAP2K4/MKK4 expression in pancreatic cancer: genetic validation of immunohistochemistry and relationship to disease course.* Clin Cancer Res, 2004. 10(24): p. 8516-20.
69. Teng DH, Perry WL 3rd, Hogan JK, et al., *Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor.* Cancer Res, 1997. 57(19): p. 4177-82.
70. Matsuo Y, Campbell PM, Brekken RA, et al., *K-Ras promotes angiogenesis mediated by immortalized human pancreatic epithelial cells through mitogen-activated protein kinase signaling pathways.* Mol Cancer Res, 2009. 7(6): p. 799-808.
71. Massague J, *TGF-beta signal transduction*. Annu Rev Biochem, 1998. 67: p. 753-91.
72. Miyazono K, ten Dijke P and Heldin CH,*TGF-beta signaling by Smad proteins.* Adv Immunol, 2000. 75: p. 115-57.
73. Peng B, Fleming JB, Breslin T, et al., *Suppression of tumorigenesis and induction of p15(ink4b) by Smad4/DPC4 in human pancreatic cancer cells.* Clin Cancer Res, 2002. 8(11): p. 3628-38.
74. Hahn SA, Schutte M, Hoque AT, et al., *DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1.* Science, 1996. 271(5247): p. 350-3.

75. Blackford A, Serrano OK, Wolfgang CL, et al., *SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer.* Clin Cancer Res, 2009. 15(14): p. 4674-9.
76. Tascilar M, Skinner HG, Rosty C, et al., *The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma.* Clin Cancer Res, 2001. 7(12): p. 4115-21.
77. Iacobuzio-Donahue CA, Fu B, Yachida S, et al., *DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer.* J Clin Oncol, 2009. 27(11): p. 1806-13.
78. Goggins M, Shekher M, Turnacioglu K, et al., *Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas.* Cancer Res, 1998. 58(23): p. 5329-32.
79. Yu L, Hebert MC, and Zhang YE, *TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses.* Embo J, 2002. 21(14): p. 3749-59.
80. Janda E, Lehmann K, Killisch I, et al., *Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways.* J Cell Biol, 2002. 156(2): p. 299-313.
81. Brown JD, DiChiara MR, Anderson KR, et al., *MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells.* J Biol Chem, 1999. 274(13): p. 8797-805.
82. Apelqvist AH, Li L, Sommer A, et al., *Notch signalling controls pancreatic cell differentiation.* Nature, 1999. 400(6747): p. 877-81.
83. Siveke JT, Lubeseder-Martellato C, Lee M, et al., *Notch signaling is required for exocrine regeneration after acute pancreatitis.* Gastroenterology, 2008. 134(2): p. 544-55.
84. Jensen J, Heller RS, Funder-Nielsen T, et al., *Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation.* Diabetes, 2000. 49(2): p. 163-76.
85. Miyamoto Y, Maitra A, Ghosh B, et al., *Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis.* Cancer Cell, 2003. 3(6): p. 565-76.
86. Wang Z, Banerjee S, Li Y, et al., *Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells.* Cancer Res, 2006. 66(5): p. 2778-84.
87. Wang Z, Zhang Y, Li Y, et al., *Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells.* Mol Cancer Ther, 2006. 5(3): p. 483-93.
88. Mullendore ME, Koorstra JB, Li YM, et al., *Ligand-dependent Notch signaling is involved in tumor initiation and tumor maintenance in pancreatic cancer.* Clin Cancer Res, 2009. 15(7): p. 2291-301.
89. Hebrok M, Kim SK and Melton DA, *Notochord repression of endodermal Sonic hedgehog permits pancreas development.* Genes Dev, 1998. 12(11): p. 1705-13.
90. Apelqvist A, Ahlgren U and Edlund H, *Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas.* Curr Biol, 1997. 7(10): p. 801-4.
91. Berman DM, Karhadkar SS, Maitra A, et al., *Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours.* Nature, 2003. 425(6960): p. 846-51.

92. Rubin LL and de Sauvage FJ, *Targeting the Hedgehog pathway in cancer.* Nat Rev Drug Discov, 2006. 5(12): p. 1026-33.
93. Hebrok M, Kim SK, St Jacques B, et al., *Regulation of pancreas development by hedgehog signaling.* Development, 2000. 127(22): p. 4905-13.
94. Feldmann G, Habbe N, Dhara S, et al., *Hedgehog inhibition prolongs survival in a genetically engineered mouse model of pancreatic cancer.* Gut, 2008. 57(10): p. 1420-30.
95. Bailey JM, Swanson BJ, Hamada T, et al., *Sonic hedgehog promotes desmoplasia in pancreatic cancer.* Clin Cancer Res, 2008. 14(19): p. 5995-6004.
96. Yauch RL, Gould SE, Scales SJ, et al., *A paracrine requirement for hedgehog signalling in cancer.* Nature, 2008. 455(7211): p. 406-10.
97. Olive KP, Jacobetz MA, Davidson CJ, et al., *Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer.* Science, 2009. 324(5933): p. 1457-61.
98. Lowy AM, Fenoglio-Preiser C, Kim OJ, et al., *Dysregulation of beta-catenin expression correlates with tumor differentiation in pancreatic duct adenocarcinoma.* Ann Surg Oncol, 2003. 10(3): p. 284-90.
99. Zeng G, Germinaro M, Micsenyi A, et al., *Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma.* Neoplasia, 2006. 8(4): p. 279-89.
100. Watanabe I, Hasebe T, Sasaki S, et al., *Advanced pancreatic ductal cancer: fibrotic focus and beta-catenin expression correlate with outcome.* Pancreas, 2003. 26(4): p. 326-33.
101. Gansauge S, Gansauge F, Ramadani M, et al., *Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis.* Cancer Res, 1997. 57(9): p. 1634-7.
102. Crawford HC, Scoggins CR, Washington MK, et al., *Matrix metalloproteinase-7 is expressed by pancreatic cancer precursors and regulates acinar-to-ductal metaplasia in exocrine pancreas.* J Clin Invest, 2002. 109(11): p. 1437-44.
103. Schleger C, Verbeke C, Hildenbrand R, et al., *c-MYC activation in primary and metastatic ductal adenocarcinoma of the pancreas: incidence, mechanisms, and clinical significance.* Mod Pathol, 2002. 15(4): p. 462-9.
104. Katoh M and Katoh M, *Transcriptional mechanisms of WNT5A based on NF-kappaB, Hedgehog, TGFbeta, and Notch signaling cascades.* Int J Mol Med, 2009. 23(6): p. 763-9.
105. Katoh M and Katoh M, *Transcriptional regulation of WNT2B based on the balance of Hedgehog, Notch, BMP and WNT signals*. Int J Oncol, 2009. 34(5): p. 1411-5.
106. Romero D, Iglesias M, Vary CP, et al., *Functional blockade of Smad4 leads to a decrease in beta-catenin levels and signaling activity in human pancreatic carcinoma cells.* Carcinogenesis, 2008. 29(5): p. 1070-6.
107. Shirts BH, Burt RW, Mulvihill SJ, et al., *A population-based description of familial clustering of pancreatic cancer.* Clin Gastroenterol Hepatol, 2010.
108. Bartsch DK, Sina-Frey M, Lang S, et al., *CDKN2A germline mutations in familial pancreatic cancer.* Ann Surg, 2002. 236(6): p. 730-7.
109. de vos tot Nederveen Cappel WH, Offerhaus GJ, van Puijenbroek M, et al., *Pancreatic carcinoma in carriers of a specific 19 base pair deletion of CDKN2A/p16 (p16-leiden).* Clin Cancer Res, 2003. 9(10 Pt 1): p. 3598-605.
110. Giardiello FMJD, Brensinger AC, Tersmette C, et al., *Very high risk of cancer in familial Peutz-Jeghers syndrome.* Gastroenterology, 2000. 119(6): p. 1447-53.
111. Rogers CD, van der Heijden MS, Brune K, et al., *The genetics of FANCC and FANCG in familial pancreatic cancer.* Cancer Biol Ther, 2004. 3(2): p. 167-9.
112. Giardiello FM, Welsh SB, Hamilton SR, et al., *Increased risk of cancer in the Peutz-Jeghers syndrome.* N Engl J Med, 1987. 316(24): p. 1511-4.

113. Howes N, Lerch MM, Greenhalf W, et al., *Clinical and genetic characteristics of hereditary pancreatitis in Europe.* Clin Gastroenterol Hepatol, 2004. 2(3): p. 252-61.
114. Threadgold J, Greenhalf W, Ellis I, et al., *The N34S mutation of SPINK1 (PSTI) is associated with a familial pattern of idiopathic chronic pancreatitis but does not cause the disease.* Gut, 2002. 50(5): p. 675-81.
115. Ozcelik H, Schmocker B, Di Nicola N, et al., *Germline BRCA2 6174delT mutations in Ashkenazi Jewish pancreatic cancer patients.* Nat Genet, 1997. 16(1): p. 17-8.
116. Slater EP, Langer P, Niemczyk E, et al., *PALB2 mutations in European familial pancreatic cancer families.* Clin Genet, 2010.
117. Jones S, Hruban RH, Kamiyama M, et al*., Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene.* Science, 2009. 324(5924): p. 217.
118. Kastrinos F, Mukherjee B, Tayob N, et al., *Risk of pancreatic cancer in families with Lynch syndrome.* Jama, 2009. 302(16): p. 1790-5.
119. Kindler HL, Niedzwiecki D, Hollis D, et al., *Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303).* J Clin Oncol, 2010. 28(22): p. 3617-22.
120. Kullmann F, Hollerbach S, Dollinger MM, et al., *Cetuximab plus gemcitabine/oxaliplatin (GEMOXCET) in first-line metastatic pancreatic cancer: a multicentre phase II study.* Br J Cancer, 2009. 100(7): p. 1032-6.
121. Moore MJ, Goldstein D, Hamm J, et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* J Clin Oncol, 2007. 25(15): p. 1960-6.
122. Feliu J, Borrega P, Leon A, et al., *Phase II study of a fixed dose-rate infusion of gemcitabine associated with erlotinib in advanced pancreatic cancer.* Cancer Chemother Pharmacol, 2010.
123. Hsu CC, Herman JM, Corsini MM, et al., *Adjuvant chemoradiation for pancreatic adenocarcinoma: the Johns Hopkins Hospital-Mayo Clinic collaborative study*. Ann Surg Oncol, 2010. 17(4): p. 981-90.
124. Kalser MH and Ellenberg SS, *Pancreatic cancer. Adjuvant combined radiation and chemotherapy following curative resection*. Arch Surg, 1985. 120(8): p. 899-903.

Chapter 5

Multivariate analysis of immunohistochemical evaluation of protein expression in pancreatic ductal adenocarcinoma reveals prognostic significance for persistent Smad4 expression only

Niki A. Ottenhof, Folkert H. M. Morsink, Fiebo ten Kate, Cornelis J. F. van Noorden, G. Johan A. Offerhaus

Cellular Oncology, 2012; 35:119-126

Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with a 5-year survival rate of <5% and an average survival of only 6 months. Although advances have been made in understanding the pathogenesis of PDAC in the last decades, overall survival has not changed. Various clinicopathological and immunohistological variables have been associated with survival time but the exact role that these variables play in relation to survival is not clear. To examine how the variables affected survival independently, multivariate analysis was conducted in a study group of 78 pancreatic ductal adenocarcinomas. The analysis included clinicopathological parameters and protein expression examined by immunohistochemistry of p53, Smad4, Axl, ALDH, MSH2, MSH6, MLH1 and PMS2. Lymph node ratio <0.2 (p=0.004), tumor free resection margins (p=0.044) and Smad4 expression (p=0.004) were the only independent prognostic variables in the multivariate analysis. Expression of the other proteins examined was not significantly related to survival. Discrepancies with other studies in this regard are likely due to differences in quantification of immunohistochemical staining and the lack of multivariate analysis. It underscores the importance to standardize the methods used for the application of immunohistochemistry in prognostic studies.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with an annual mortality rate almost equaling incidence. Approximately 36.800 patients die annually from PDAC in the USA, making it the fourth leading cause of cancer-related death [1]. Five-year survival rates have not changed over the last decades and are currently still <5% [2-4]. Although advances have been made in the understanding of the pathogenesis of PDAC, these were not translated into improved prognosis [5]. Most patients present with locally advanced or distant metastatic disease, making resection with curative intent elusive. From the 5-20% of PDAC patients who qualify for resection only 10-18% will reach 5-year survival. Still, 5-year survival cannot be equated to cure because patients still die from recurrent disease after 5 years [6, 7].

Clinicopathological factors such as low disease stage [4, 8], resection margin [4, 8] and lymph node metastasis [9] have been associated with survival although the exact role of these characteristics in survival is unknown. Moreover, various tumor-specific protein expression patterns have been reported to be associated with overall survival in pancreatic cancer patients. Persistent expression of Smad4, a tumor suppressor gene affected in ~55% of PDACs, was found to be a strong prognosticator improving both disease-free and overall survival (OS) [10, 11]. Furthermore, micro-satellite instability, caused by defects in the mismatch repair (MMR) genes, has been reported to affect prognosis favorably [12] although not all studies confirm this [13, 14]. Another protein described to be associated with prognosis in PDAC is Axl, a receptor tyrosine kinase often involved in cancer development [15, 16]. Recently, a study by Rasheed et al. linked expression of aldehyde dehydrogenase (ALDH) to worse prognosis; it was suggested that ALDH-positive cells have tumor-initiating potential and that the percentage of ALDH-positive cells negatively affects OS [17]. However, in most of these studies, the clinical and histological characteristics were evaluated without adjustment for other variables that affect prognosis through a multivariate analysis.

To examine whether the different clinical and histological factors affect survival independently, a multivariate analysis was therefore conducted on a cohort consisting of 78 PDACs. The variables included clinicopathological parameters and expression patterns of most of the proteins previously reported to have a role in PDAC survival. The following proteins were examined: p53, Smad4, Axl, ALDH, and four mismatch repair genes; MSH2, MSH6, MLH1 and PMS2.

Methods

Patient selection

Paraffin-embedded tissue from 78 primary infiltrating PDACs was obtained from the Surgical Pathology archives of 3 cancer treatment centers: the University Medical Center Utrecht, the Academic Medical Center Amsterdam and the Erasmus Medical Center Rotterdam. Clinical data that was obtained included age, sex, tumor size, TNM-stage, histological grade, lymph-node status and exact survival time in months for all patients. Data were not available with respect to treatment of the patients or time of recurrence of the tumors and could therefore not be linked with the parameters investigated in this study.

Tissue micro arrays (TMAs)

TMAs were developed using formalin fixed paraffin embedded tissue as previously described [18, 8]. Briefly, for each case representative areas containing neoplastic cells were marked on a hematoxylin-eosin stained sections which served as template. Three 0.6 mm cores were punched from the donor block and injected into the receiver block. For each patient, a 0.6 mm core from a non-neoplastic lymph node was included as control tissue.

Antibody	**Company**	**Dilution**	**Incubation time**	**Substrate**
p53 (BP53-12) #MS-738-7	Thermo Scientific, Fremont, CA, USA	1:2000	1hr, room temperature	DAB
Smad4 (B-8) #sc-7966	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:300	1hr, room temperature	DAB+
ALDH #61195	BD Transduction Laboratories, Franklin Lakes, NJ, USA	1:200	1hr, room temperature	DAB
Axl #AF154	R&D Systems, Minneapolis, MN, USA	1:100	1hr, room temperature	DAB
MLH1 #13271A	BD **Pharmingen**, San Diego, CA, USA	1:50	Overnight, 4C	DAB+
MSH2 # NA27	Oncogene Research Products, Schwalbach, Germany	1:200	Overnight, 4C	DAB+
MSH6 # 610919	BD Transduction Laboratories	1:200	Overnight, 4C	DAB+
PMS2 #556415	BD Transduction Laboratories	1:500	Overnight, 4C	DAB+

Table 1 Antibodies used for immunohistochemistry.

Immunohistochemistry (IHC)

IHC was performed on 4 µm-thick sections of the TMAs to analyse expression of p53, Smad4, Axl, ALDH and the four MMR proteins MSH2, MSH6, MLH1 and PMS2. Sections were deparafinized using routine techniques. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min after which sections were pretreated if necessary with ARS pH 9 for 10 minutes in the autoclave and cooled for 10 minutes. Before primary antibody application, sections

were incubated with Protein Block Serum-Free (Dako Cytomation, Carpenteria, CA, USA). Then primary antibodies were applied. Antibody binding was visualized using the PowerVision+ Poly-HRP kit (Immunologic, Duiven, The Netherlands) with 3,3-diaminobenzidin (DAB; Sigma-Aldrich, Seelze, Germany) or DAB+ (Dako Cytomation) as chromogen. Sections were counterstained with hematoxylin and cover slips were applied. The primary antibodies that were used, their dilution and incubation time are described in Table 1.

IHC labeling was scored by a single investigator after consensus was reached about cut off levels with an experienced pathologist behind a multiheaded microscope. In case of doubt, sections were again evaluated with the pathologist. Scoring was then based on consensus after discussion. The scoring method differed per antibody. P53 staining was scored as positive when nuclear accumulation of the protein was observed in more than 80% of the cancer cells. Absent or diffuse weak staining was scored as negative, indicating normal expression of p53. Smad4 and the MMR proteins were scored as negative when labeling was absent in the neoplastic cells. Weak or strong labeling was scored as positive. Axl staining was scored as negative when labeling was observed in 0-10% of the neoplastic cells. Slides showing labeling in >10% of the neoplastic cells were scored as positive. Scoring methods used in this study correspond with previously published papers [15, 19, 20]. For ALDH, scoring was performed in two different ways. First, sections were scored in a similar way as was described for Axl. The second way to score ALDH staining was according to the method reported by Rasheed et al. [17]. Only tumors exhibiting intense staining in both nuclei and cytoplasm of neoplastic cells, that was at least 2-fold higher than staining in normal pancreatic acinar cells adjacent to the tumor, were scored as positive (Figure 1). In most cases the 3 tissue cores showed similar staining intensity. In some cases, staining intensity differed between the cores. However, scoring was performed per tumor, not per core. Therefore, the 3 cores were evaluated as a whole and the IHC score was based on the total cancer cell population in the 3 cores.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 (SPSS, Chicago, IL, USA). Associations between the different variables were examined using the Pearson's Chi Square test. The survival was estimated by the Kaplan-Meier method and tested by log-rank test for statistical significance. Kaplan-Meier graphs were plotted using GraphPad Prism (GraphPad, La Jolla, CA USA). To evaluate correlations between the different variables, a Pearson's correlation coefficient was calculated for both clinical characteristics and protein expression patterns. The variables with prognostic potential in the univariate analyses ($p \leq 0.10$) and the variables that correlated significantly with one of the parameters were included in

the multivariate analysis. Variables that did not correlate significantly were removed in a step-wise manner. The Cox proportional hazards model was used. A p-value <0.05 was considered to indicate statistical significance.

Results

Clinical characteristics

The mean age of the 78 patients was 63 (range, 40-77). The diagnosis PDAC was confirmed in all patients. The median overall survival was 27 months. Twelve patients (15%) reached 5-year survival, 2 patients (3%) reached 10-year survival. Three patients presented with PDAC in the tail of the pancreas. The other 75 were diagnosed with a tumor in the head of the pancreas. Sixty-nine patients underwent a Whipple's pancreatoduodenectomy. Seven other patients underwent either a pylorus-preserving pancreatoduodenectomy (n=6) or a complete pancreatoduodenectomy (n=1). Two patients underwent corpus/tail resection. Operation procedures did not affect survival (p=0.48). Demographics and tumor characteristics are listed in Table 2. Univariate analysis revealed no differences in survival time related to age, gender, tumor size or histological grade. Absence of lymph node metastasis and a tumor-free resection margin showed a borderline significant correlation with improved survival (p=0.07 and p=0.06, respectively). The variables significantly improving survival as was shown by univariate analysis were lymph node ratio (LNR) (OS LNR<0.2, 34.5 months; LNR>0.2, 15.6 months; p=0.002) and tumor stage (OS stage I or IIA, 43.1 months; stage IIB or III, 21.0 months; p=0.02) (Figure 2).

Immunohistochemical evaluation

We determined protein expression by immunohistochemistry for 8 different proteins. Strong nuclear staining of p53, indicating a defect in the protein, was found in 54% of the cases. Smad4 expression was completely absent in 43% of the cases. Staining of the mismatch repair proteins MSH-2, MSH-6, PMS-2 and MLH-1 was unaffected in 95%, 97%, 93% and 99%, of the cases, respectively. Axl expression was observed in 22% of the tumors. The other tumors were Axl negative. Although 75% of the tumors exhibited positive staining of ALDH protein, high intensity staining of ALDH protein as described by Rasheed et al. [17] was observed in only 11 tumors (16%).

Univariate analysis revealed Smad4 expression as a strong prognostic variable for survival (p=0.008) (Table 3). The other variables did not have a statistically significant prognostic value.

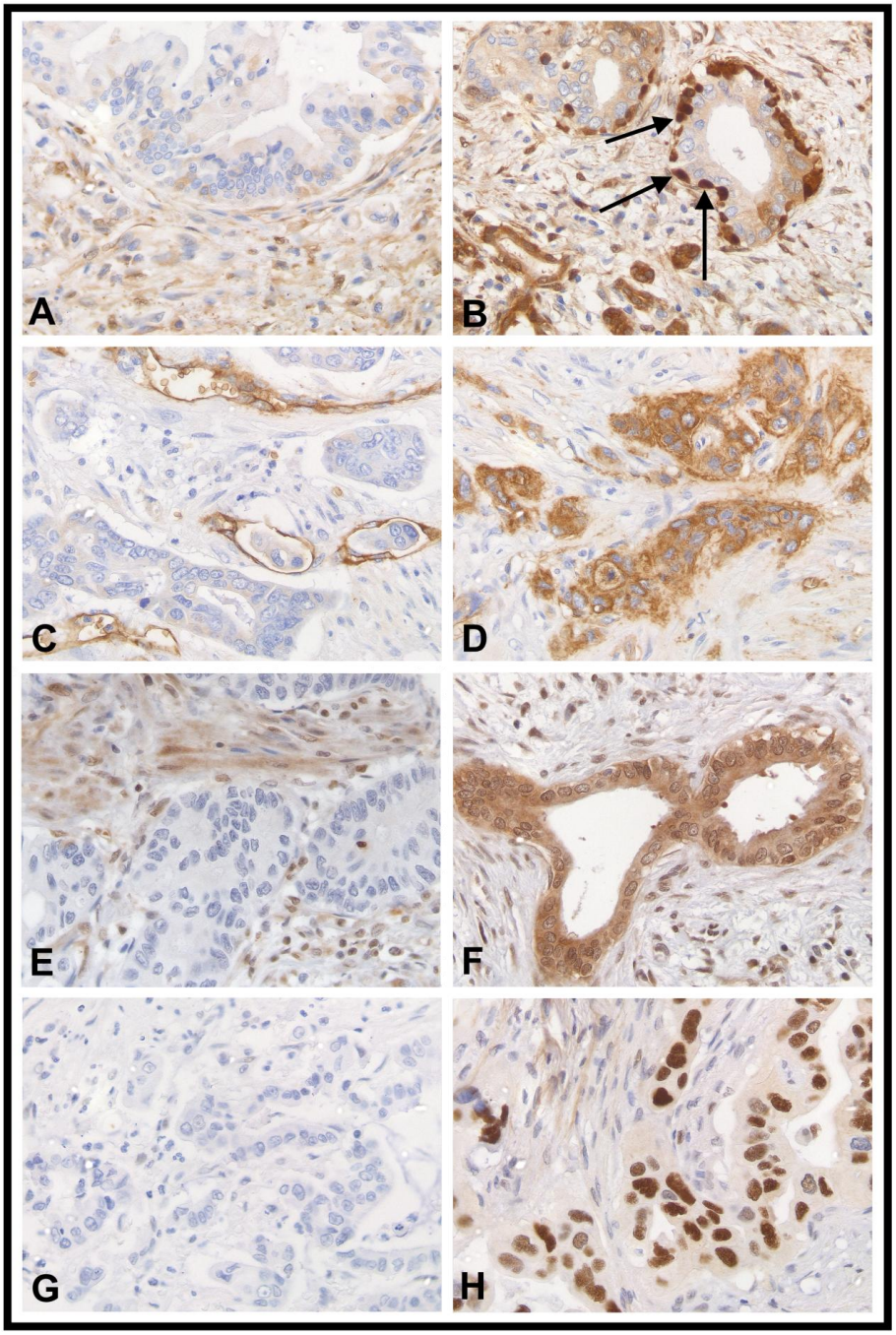


Figure 1 Immunohistochemical evaluation of both negative (A,C,E,G) and positive (B,D,F,H) expression of ALDH (A/B), Axl (C/D), Smad4 (E/F) and p53 (G/H). Arrows: intense staining of ALDH in the basally located neoplastic cells.

Variable	Total (n=78)	Median survival (months)	95% CI	p value (log-rank test)
Demographics				
Age (%)				0.69
< 65 years	39 (50%)	24.8	13.9-35.8	
≥ 65 years	39 (50%)	28.5	27.5-39.4	
Gender				0.76
male	35 (45%)	23.6	15.6-31.5	
female	43 (55%)	29.2	16.8-41.6	
Tumor characteristics				
Tumor size (cm) (1 case missing)				0.23
<2,0	13 (17%)	43.8	9.0-78.6	
≥2,0	64 (83%)	22.7	16.7-28.7	
Histological grade (1 case missing)				0.81
poor	21 (27%)	23.8	8.5-39.1	
moderate	40 (52%)	27.9	16.2-39.7	
well	16 (21%)	28.7	15.7-41.7	
Stage				0.02*
I or /IIA	20 (26%)	43.1	24.2-62.1	
IIB or III	58 (74%)	21.0	13.4-28.6	
Lymph node status (1 case missing)				0.07
N0	23 (43%)	38.7	21-56	
N1	54 (57%)	16.8	14-30	
Lymph node ratio (1 case missing)				0.002*
<0.2	46 (60%)	34.5	22.6-46.4	
≥0.2	31 (40%)	15.6	9.2-22.1	
Resection margin				0.06
R0	52 (67%)	31.6	22.6-46.4	
R1	26 (33%)	15.6	9.2-22.1	

Table 2 Distribution of demographic and tumor-related factors and univariate survival analysis for 78 PDAC patients. * Significant correlation with survival.

Multivariate analysis

To evaluate the correlation between the different variables, Pearson's correlation coefficient was calculated for both the clinical characteristics and protein expression patterns. Tumor stage was significantly correlated with lymph node ratio

(p<0.001) and resection margin (p=0.04). There was a strong correlation between expression of MMR proteins. MSH2 and MSH6 expression were strongly correlated (p=0.005), as were PMS2 and MLH1 expression (p<0.001).

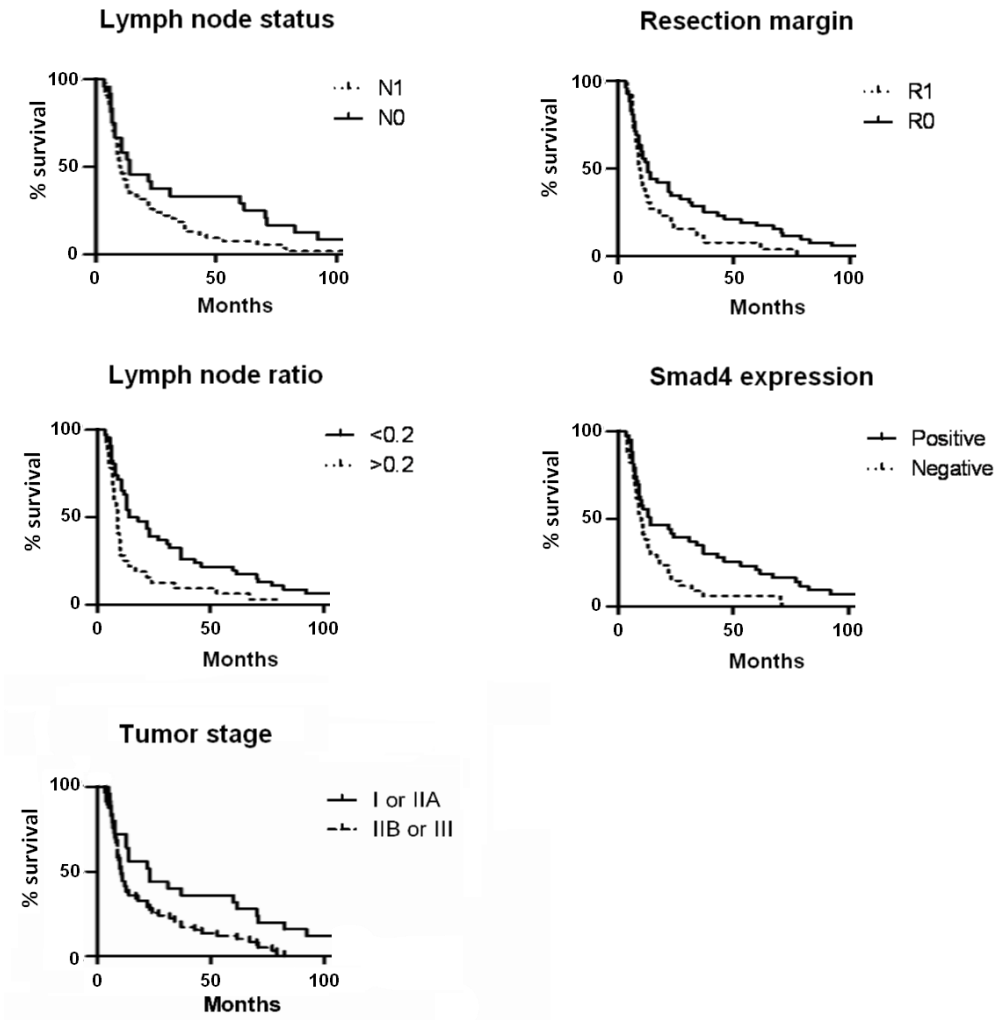


Figure 2 Kaplan-meier plots of overall survival of 78 PDAC patients in relation to node status (log-rank p=0.066), resection margin (log-rank p=0.056), lymph node ratio (log-rank p=0.002), Smad4 expression (log-rank p=0.008) and tumor Stage (log-rank p=0.02).

The variables that had prognostic potential in the univariate analysis (p≤0.10) or that were significantly correlated with one of the variables were subjected to multivariate analysis. The analysis included the variables lymph node status, LNR, resection margin, tumor stage and Smad4 expression. LNR<0.2, a tumor-free

resection margin and persistent Smad4 expression significantly favored survival in multivariate analysis as shown in Table 4.

Variable	**Total**	**Median survival**	**95% CI**	**p value (log-rank test)**
Protein expression				
P53				0.84
negative	31 (46%)	30.1	14.1-46.0	
nuclear	36 (54%)	25.8	16.5-25.1	
Smad4				0.008*
negative	34 (44%)	15.8	10.4-21.2	
positive	49 (56%)	35.6	22.9-48.4	
Axl				0.40
negative	54 (78%)	23.6	15.6-31.5	
positive	15 (22%)	29.2	16.8-41.6	
ALDH (diffuse staining)				0.12
negative	18 (84%)	15.3	8.1-22.5	
positive	54 (16%)	28.6	19.1-38.1	
ALDH (high-expression)				0.11
negative	58 (84%)	22.7	16.7-28.7	
positive	11 (16%)	43.8	9.0-78.6	
MSH2				0.45
negative	4 (5%)	30.4	6.6-54.2	
positive	69 (95%)	25.15	16.2-39.7	
MSH6				0.73
negative	2 (3%)	35.2	0-86.6	
positive	70 (97%)	25.0	17.5-32.6	
MLH1				0.98
negative	1 (1%)	17.0	17.0-17.0	
positive	72 (99%)	25.7	18.3-33.2	
PMS2				0.41
negative	5 (7%)	15.2	3.9-26.6	
positive	70 (93%)	27.7	19.2-36.2	

Table 3 Univariate analysis for histological factors in 78 PDAC patients. * Significant correlation with survival.

Variable		Hazard ratio	95% CI	p value
Tumor resection margin	R0	1.00		
	R1	1.86	1.02-3.41	0.044*
Lymph node ratio	N0	1.00		
	N1	2.36	1.37-4.07	0.002*
Smad4	negative	1.00		
	positive	2.34	1.30-4.21	0.004*

Table 4 Multivariate analysis including: tumor stage, lymph node status, lymph node ratio, resection margin, tumor stage and Smad4 expression.

Discussion

Although tremendous progress has been made over the last decades in the understanding of the pathogenesis of PDAC, PDAC patients still die within a few months after diagnosis. Critical analysis of factors involved in survival and prognosis potentially leads to a better understanding of the pathogenesis of PDAC. Therefore, identification of patient- and/or tumor-specific characteristics associated with increased survival time is an important strategy. Although multiple studies have been conducted concerning the prognostic significance of different variables, the picture remains incomplete and unclear. Tumor size, lymph node involvement, resection margin and histological grade have all been reported to significantly affect survival time (4, 8), although inconsistencies between studies remain [6, 21]. At present, only LNR was found to be a significant prognosticator in every study it was evaluated in [6, 9, 21, 22].

Apart from the different demographics, immunohistochemical evaluation of protein expression revealed multiple potential prognosticators in PDAC in recent publications [11, 15, 21-23]. However, multivariate analysis to evaluate whether these proteins have independent prognostic significance was generally not performed. In order to evaluate results from earlier publications on the prognostic relevance of clinical characteristics and to determine the role that various proteins play in prolonged survival, we performed multivariate analysis including most characteristics that have been implicated to be involved. Most importantly, we tried to identify which proteins remained significant for prognosis in multivariate analysis.

In the current study, LNR<0.2 was the strongest prognosticator which confirms results from other studies [23-25]. Another important prognostic factor that was described previously, persistent Smad4 expression [10, 11], was also a significantly favorable prognosticator with respect to survival in our cohort and remained so in multivariate analysis.

Positive Axl expression in PDAC has been related to shorter overall survival [15, 16]. Unfortunately, multivariate analysis was not performed by Koorstra et al. [15]. Song et al. [16] performed multivariate analysis including both Axl expression and lymph node involvement which resulted in a marginally significant effect of Axl on survival time. However, this study included only stage II PDACs making comparison with our results difficult. We did not detect a correlation between either Axl expression and lymph node status or Axl expression and survival time. Furthermore, only 23% of the tumors expressed Axl, which is a much lower proportion than described previously. It is possible that the scoring method for Axl expression in our study differed, leading to a smaller percentage of Axl-positive tumors. Because of these contradictory findings on Axl expression in PDAC, conclusions on the prognostic relevance of Axl expression cannot be drawn and it seems advisable to further delineate the role of Axl in PDAC development and progression.

Another group of proteins reported to affect survival are the MMR proteins MSH2, MSH6, MLH1 and PMS2. Mutations or epigenetic changes in these genes lead to microsatellite instability. Microsatellite instable tumors have been claimed to have a significantly better prognosis than their microsatellite stable counterparts [12, 13]. Although we did not assess microsatellite instability, we evaluated MMR protein activity using IHC. Similar to previously published data, approximately 13% of the tumors showed absence of expression of one or more of the MMR proteins [12, 26]. There was a strong correlation between the expression of the different mismatch repair proteins. MSH2 and MSH6 expression were strongly correlated, as were MLH1 and PMS2 expression. This was expected as both MSH2/MSH6 and MLH1/PMS2 form heterodimers through which they function in the repair of DNA mismatches [27]. However, we found no significant relationship between MMR protein expression and survival.

Recent reports on ALDH expression in malignancies focused on a small proportion of the cancer cell population (approximately 1% of the total tumor volume) which is characterized by a higher tumorigenic potential. These so-called ‘tumor-initiating cells’ have been under investigation in the last decade and a study on these ALDH-expressing ‘tumor-initiating cells’ in PDAC and the prognostic significance of their presence was recently published [17]. Although IHC revealed a large proportion of neoplastic cells exhibiting ALDH expression, Rasheed et al [17] focused only on the cells that showed strong nuclear labeling of ALDH with a 2-fold or higher intensity as compared to the normal acinar cells. This resulted in a small percentage of ALDH-high tumors with significant prognostic potential. When scoring for expression of ALDH in our cohort, 75% of the tumors were positive, albeit with variable intensity. There was no relation between ALDH expression and

survival time. When using the stringent scoring requirements as suggested by Rasheed et al [17], the percentage of ALDH-high tumors was 16%. Again, we did not find a correlation between high ALDH expression tumors and survival time. Because cancer research is focused on 'tumor-initiating cells', it seems advisable to further investigate IHC staining of ALDH protein as our study demonstrates that discrepancies remain between the various studies.

IHC for the evaluation of protein expression is a fast and cheap method with great value in the laboratory. For example, IHC of Smad4 accurately mirrors Smad4 expression [28] and IHC demonstrates p53 defects as nuclear-bound protein. However, for most proteins there is not a standard method available for scoring IHC stained tumors and this makes interpretation and comparison of studies cumbersome. In the current study, this was illustrated in the ALDH expression analysis, where the two different scoring methods resulted in a different percentage of ALDH-positive tumors. Effort should be put into standardization of IHC scoring to increase the accuracy of evaluating protein expression using IHC to obtain valid quantitative data [29].

In conclusion, this study confirmed the prognostic significance of LNR and resection margin in PDAC in multivariate analysis. These two characteristics, together with Smad4 expression, had a significant effect on survival time and should be considered when determining patient specific prognosis. The expression patterns of the other proteins investigated had no significant relation with overall survival time in the multivariate analysis. Most of them have been reported to affect survival in other studies published, but more research has to be performed before a definitive conclusion can be drawn concerning the value of these proteins in determining prognosis.

Reference list

1. Jemal A, Siegel R, Ward E, et al., *Cancer statistics, 2009.* CA Cancer J Clin 59: 225-49, 2009.
2. Maitra A, Kern SE and Hruban RH, *Molecular pathogenesis of pancreatic cancer.* Best Pract Res Clin Gastroenterol 20: 211-26, 2006.
3. Schneider G, Siveke JT, Eckel F et al., *Pancreatic cancer: basic and clinical aspects.* Gastroenterology 128: 1606-25, 2005.
4. Hsu CC, Herman JM, Corsini MM, et al., *Adjuvant chemoradiation for pancreatic adenocarcinoma: the Johns Hopkins Hospital-Mayo Clinic collaborative study.* Ann Surg Oncol 17: 981-90, 2010.
5. David M, Lepage C, Jouve JL, et al., *Management and prognosis of pancreatic cancer over a 30-year period.* Br J Cancer 101: 215-8, 2009.
6. Schnelldorfer T, Ware AL, Sarr MG, et al., *Long-term survival after pancreatoduodenectomy for pancreatic adenocarcinoma: is cure possible?* Ann Surg 247: 456-62, 2008.
7. Ferrone CR, Brennan MF, Gonen M, et al., *Pancreatic adenocarcinoma: the actual 5-year survivors*. J Gastrointest Surg 12: 701-6, 2008.
8. Barugola G, Partelli S, Marcucci S, et al., *Resectable pancreatic cancer: who really benefits from resection?* Ann Surg Oncol 16: 3316-22, 2009.
9. Katz MH, Wang H, Fleming JB, et al., *Long-term survival after multidisciplinary management of resected pancreatic adenocarcinoma*. Ann Surg Oncol 16: 836-47, 2009.
10. Hahn SA, Schutte M, Hoque AT, et al., *DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1.* Science 271: 350-3, 1996.
11. Blackford A, Serrano OK, Wolfgang CL, et al., *SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer.* Clin Cancer Res 15: 4674-9, 2009.
12. Nakata B, Wang YQ, Yashiro M, et al., *Prognostic value of microsatellite instability in resectable pancreatic cancer.* Clin Cancer Res 8: 2536-40, 2002.
13. Goggins M, Offerhaus GJ, Hilgers W, et al., *Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+.* Am J Pathol 152: 1501-7, 1998.
14. Maple JT, Smyrk TC, Boardman LA, et al., *Defective DNA mismatch repair in long-term (> or =3 years) survivors with pancreatic cancer.* Pancreatology 5: 220-7; discussion 227-8, 2005.
15. Koorstra JB, Karikari CA, Feldmann G, et al., *The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target.* Cancer Biol Ther 8: 618-26, 2009.
16. Song X, Wang H, Logsdon CD, et al.,*Overexpression of receptor tyrosine kinase Axl promotes tumor cell invasion and survival in pancreatic ductal adenocarcinoma.* Cancer, 2010. 15;117(4):734-43.
17. Rasheed ZA, Yang J, Wang Q, et al., *Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma.* J Natl Cancer Inst 102: 340-51, 2010.
18. Milne AN, Sitarz R, Carvalho R, et al., *Early onset gastric cancer: on the road to unraveling gastric carcinogenesis.* Curr Mol Med 7: 15-28, 2007.
19. Maitra A, Adsay NV, Argani P, et al., *Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray.* Mod Pathol 16: 902-12, 2003.

20. Bergmann F, Aulmann S, Wente MN, et al., *Molecular characterisation of pancreatic ductal adenocarcinoma in patients under 40*. J Clin Pathol 59: 580-4, 2006.
21. Butturini G, Stocken DD, Wente MN, et al., *Influence of resection margins and treatment on survival in patients with pancreatic cancer: meta-analysis of randomized controlled trials.* Arch Surg 143: 75-83; discussion 83, 2008.
22. Bhatti I, Peacock O, Awan AK, et al., *Lymph node ratio versus number of affected lymph nodes as predictors of survival for resected pancreatic adenocarcinoma.* World J Surg 34: 768-75, 2010.
23. Riediger H, Keck T, Wellner U, et al., *The lymph node ratio is the strongest prognostic factor after resection of pancreatic cancer.* J Gastrointest Surg 13: 1337-44, 2009.
24. Pawlik TM, Gleisner AL, Cameron JL, et al., *Prognostic relevance of lymph node ratio following pancreaticoduodenectomy for pancreatic cancer.* Surgery 141: 610-8, 2007.
25. Showalter TN, Winter KA, Berger AC, et al., *The influence of total nodes examined, number of positive nodes, andlLymph node ratio on survival after surgical resection and adjuvant chemoradiation for pancreatic cancer: A secondary analysis of RTOG 9704.* Int J Radiat Oncol Biol Phys, 1;81(5):1328-35, 2011.
26. Ghimenti C, Tannergard P, Wahlberg S, et al., *Microsatellite instability and mismatch repair gene inactivation in sporadic pancreatic and colon tumours.* Br J Cancer 80: 11-6, 1999.
27. Kunkel TA and Erie DA: *DNA mismatch repair.* Annu Rev Biochem 74: 681-710, 2005.
28. Wilentz RE, Su GH, Dai JL, et al., *Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas : a new marker of DPC4 inactivation.* Am J Pathol 156: 37-43, 2000.
29. Chieco P, Jonker A and Van Noorden CJF, *Image Cytometry. 1st edn.* BIOS Scientific Publishers, Oxford, 2001.

Chapter 6

Genetic and proteomic analysis of very long-term survivors (>10 year) of pancreatic cancer

Niki A. Ottenhof, Athanasios Tsiatis, Alexis Norris, Keith Jackson, Michael Havez, David Klimstra, James Eshleman, N. Volkan Adsay, G. Johan A. Offerhaus, Ralph Hruban, Anirban Maitra

Manuscript in preparation

Abstract
Pancreatic ductal adenocarcinoma (PDAC) remains a malignancy with one of the worst prognoses. Moreover, survival and treatment options have not improved much in recent years. Only approximately 5% of PDAC patients reach 5 year survival and even then recurrence often occurs indicating that it may be impossible to cure PDAC. A wide range of clinical characteristics has been related to a better prognosis of PDAC. Although these factors can be used to estimate the prognosis in general, they do not apply to every case. Some patients survive against odds. Therefore, a cohort of PDAC patients was formed consisting of these very rare long-term survivors. The databases of three specialized pancreatic cancer treatment centres were used to form a cohort with solely ≥10 year survivors of PDAC. The histological samples of 24 patients were re-evaluated and the existence of PDAC survivors was confirmed. After grouping the cohort with a comparable control group of 10 short-term survivors (6-18 months), several genetic and proteomic features were evaluated in search for the tumor- or patient-specific characteristics that were responsible for this long-term survival. *KRAS2* mutation analysis revealed a comparable mutation spectrum as found in the short-term survival cohort and the general PDAC population. The same was observed with immunohistochemical analysis of p53 and hENT1/dCK expression. Only Smad4 expression revealed a slightly higher percentage of the wild-type genotype in the long-term survivor group compared to the general population. Still, this observation can only marginally explain the longer survival of a few cases. We conclude that very long-term survivors of PDAC exist, albeit in only a very small percentage (<0.1%). Furthermore, we did not find any difference in the parameters that we investigated between long-term and short-term survivors. Nevertheless, further investigations of these very long-term survivors of PDAC and expanding the literature on this unique cohort may still hold valuable clues to improve PDAC patients survival.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a very dismal prognosis and is currently still responsible for the fourth largest number of cancer-related deaths in the United States [1]. With a 5-year survival of less than 5% and a median survival of only 6 months, it is one of the most devastating types of cancer [2, 3]. Although many advances have been made over the last decades in understanding the pathogenesis of this disease, very few treatment options have been shown to improve this poor prognosis [4]. Most patients still present with locally advanced or distant metastatic disease, making resection with curative intent elusive. Only 5-20% of PDAC patients qualify for resection, but only approximately 10% of these patients will reach 5-year survival. Still, this 5-year survival cannot be equated to cure because 16-50% of these patients die from recurrent disease [5, 6]. Recurrence has been described long after the initial diagnosis indicating that PDAC might not be curable [7].

A wide range of clinicopathological characteristics have been described that are related to a better prognosis of PDAC, such as absence of lymph node metastasis, tumor-free resection margins (R0), small tumor size and a low disease stage at diagnosis [5, 6, 8, 9]. In addition to these conventional clinicopathological features, recent reports on PDAC show that microsatellite instability, high hENT1 expression in combination with gemcitabine treatment and high dCK expression all may significantly improve the prognosis [10-16]. Still, it is not clear yet what characteristics affect tumor aggressiveness.

Although the above-mentioned features are useful when determining patient-specific prognosis, they do not apply to every case. Some patients survive against odds. The present study focuses on these very long-term survivors. In an attempt to address the question what makes these patients survive despite their grim outlook, a cohort of very long-term survivors of PDAC was formed. Because recurrence can occur long after resection, a cut-off point of 10-year survival was selected. This cohort of very long-term survivors was composed using the Johns Hopkins University (JHU) database and the Memorial Sloan Kettering database with additional samples from the Emory University. All ≥10-year survivors of PDAC were selected and reviewed. After grouping this cohort with a comparable control group of short-term survivors (6-18 months), several genetic and proteomic features were evaluated in search for the tumor- or patient-specific characteristics that enabled this long-term survival.

Materials and methods

Patient selection

The entire databases of the JHU, the Memorial Sloan Kettering and the Emory University were scanned for ≥10-year survivors of PDAC.

In the JHU archive, 42 cases were found. Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained when available. Archival tissue of 16 patients was not available and these patients were excluded. The remaining 26 cases were reviewed by 2 highly-trained pathologists, specialized in PDAC. This cohort was expanded using cases from Emory University, Atlanta, GA, and cases from the

Memorial Sloan Kettering, New York, NY, using a similar process of selection. After excluding cases that where either not diagnosed directly to be PDAC or had a PDAC with favorable prognostic features, the total research cohort consisted of 24 ≥10-year survivors of PDAC.
A control group of short-term survivors was also assembled. Survival <18 months was defined as short-term survival. Patients who died within 6 months after resection were excluded to prevent inclusion of patients who had died from complications of surgery. The cases that met the inclusion criteria were matched with our cohort on the basis of age, tumor stage, lymph node ratio and grade of differentiation: all factors known to affect prognosis. In total, 10 control cases were selected from the JHU database. Further histological analysis was performed on all cases.

Laser capture microdissection (LCM)

Four micron-thick sections of FFPE tissue were mounted on UV-pretreated PALM membrane slides (Carl Zeiss, Jena, Germany). Sections were deparafinized and stained with hematoxilin and eosin (H&E). The slides were placed on ice and immediately used for LCM. An H&E-stained section of every case was used as reference. A highly-trained pathologist encircled the neoplastic regions in these reference sections which were used during the LCM to navigate through the sections on the PALM membrane slides. Neoplastic cells were dissected from the designated areas by LCM using the PALM cold laser (Carl Zeiss) and a computer displaying the microscopic view. Once the neoplastic cells were cut loose, the tissue was beamed into the cap of a 0.5 ml tube containing 40 µl of diethylpyrocarbonate (DEPC)-treated water (Figure 1).

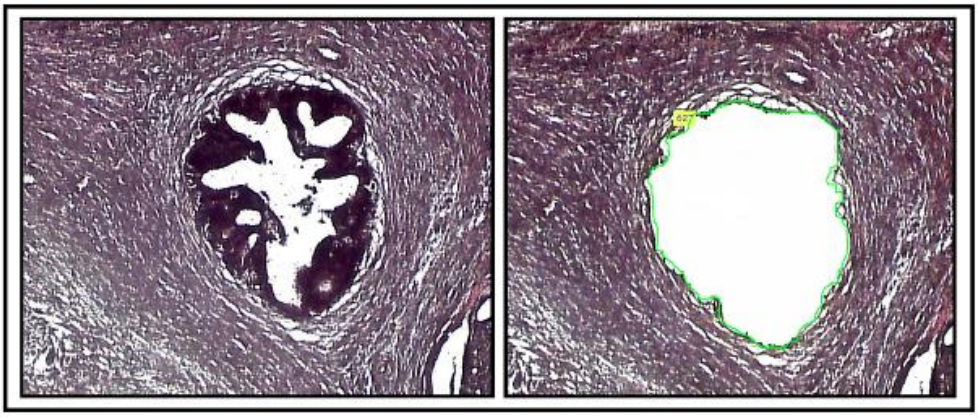


Figure 1 Laser capture microdisscetion using a PALM microbeam. After selection of the neoplastic area indicated by the green line, the area was dissected by cold laser and beamed into a 0.5 ml tube.

DNA isolation

After a sufficient amount of tissue was obtained with LCM, DNA was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Valencia CA, USA) according to the protocol provided by the manufacturer. To quantify the total amount of amplifiable

DNA, the Quantifiler kit (Applied Biosystems, Foster City CA, USA) was used according to the manufacturer's protocol. In short, standard dilutions were made from the concentrated control provided with the kit. A master mix was prepared containing primer and enzyme mix. One microliter of isolated DNA was added to the mastermix. All standards and samples were run in duplicates and 2 dH_2O controls were included. The polymerase chain reaction (PCR) was run on the StepOnePlus RealTime PCR system (Applied Biosystems).

Pyrosequencing for KRAS codon 12/13

Samples were PCR amplified using the *KRAS* v2.0 kit (Qiagen) according to the manufacturer's protocol as previously described [17]. In short, a reaction mix (25 µl final volume) was prepared containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 5 pmol of biotinylated forward primer, and 5 pmol of biotinylated reverse primer, 0.8 Units of HotStar TaqDNA polymerase (Qiagen), 2 µl template DNA and dH_2O. Cycling conditions were as follows: 95°C 15 minutes, 48X (95°C 20 seconds, 53°C 30 seconds, 72°C 20 seconds), 72°C 5 minutes, 8°C hold. Following amplification, 10 µl of biotinylated PCR product was immobilized on streptavidin-coated sepharose beads (streptavidin sepharose high performance; GE Healthcare Bio-Sciences, Piscataway NJ, USA) and washed in 70% ethanol. The purified biotinylated PCR product was released into the PyroMark Q2 (Biotage, Uppsala, Sweden) with PyroMark Gold reagents (Qiagen) containing 0.3 µM sequencing primer and annealing buffer. The nucleotide dispensation order for codons 12/13 of *KRAS* was: 5-TACGACTCAGATCGTAG-3.

Microsatellite instability (MSI) analysis

MSI analysis was performed using the Promega MSI analysis system, version 1.2 (Promega, Madison WI, USA) according to manufacturers' protocol as previously described [18, 19]. Five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) were used for MSI determination. MSI at ≥2 mononucleotide loci was marked as MSI-high, instability at a single mononucleotide locus as MSI-low, and no instability at any of the loci tested as microsatellite stable. Cycling conditions used were 95°C 11 minutes, 96°C 1 minute, 16X (94°C 30 seconds, 58°C 30 seconds, 70°C 1 minute), 32X (90°C 30 seconds, 58°C 30 seconds, 70°C 1 minute), 60°C 30 minutes, 4°C hold. Products were analyzed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems).

Immunohistochemistry

Immunohistochemistry of p53, Smad4, human equilibrative nucleoside transporter 1 (hENT1) and deoxycytidine kinase (dCK) was performed on FFPE tissue. Sections were deparafinized by routine techniques. Endogenous peroxidases were blocked with 3% H_2O_2 for 5-10 min. Sections were pretreated as described in Table 1. Incubations with the primary antibodies lasted for 30 min to 1 hour at room temperature. Bound antibody attachment was visualized using the streptavidin-

HRP solution kit (DakoCytomation, Carpenteria CA, USA) for 30 minutes. DAB+ (DakoCytomation) was used as chromogen. Sections were counterstained with hematoxilin and a cover slip was applied. Antibodies and dilutions are specified in Table 1.

Antibody	Clone	Source	Dilution	Pre-treatment
p53	BP53-11	Ventana, Tucson AZ, USA	Pre-diluted	Cell conditioning 1 (Ventana)
Smad4	B-8 (sc-7966)	Santa Cruz, Santa Cruz CA, USA	1:500	Bond Enzyme 1 (Leica)

Table 1 Antibodies and incubation conditions used for immunohistochemistry.

Immunohistochemical labeling was scored by 2 highly-trained pathologists, specialized in PDAC. Consensus was reached in all cases. Intensity of labeling was designated as absent, weak or strong. Labeling intensity was only designated as weak/strong when more than 80% of the neoplastic cells showed this intensity. Scoring of the p53 labeling differed from the other scoring system because strong nuclear staining indicates a defective p53 gene which was therefore scored as absent. Non-malignant pancreatic ductal cells present in the sections were used as a control.

Results

Patients

The 26 patients that were found using the JHU database were diagnosed between 1989 and 1998. Several patients were excluded because their tumors showed features of medullary (n=1), ampullary (n=3) or billiary (n=3) origin. All pre-invasive lesions were excluded (n=4). One tumor showed colloid features, which is known to have a better prognosis, and was therefore excluded. The remaining study cohort consisted of 14 patients. A similar process for patient selection was performed at the Memorial Sloan Kettering (7 cases) and the Emory University (3 cases) completing the total study cohort at 24 cases.

Clinical characteristics

Average age at resection was 63 years, with 62.5% of the patients being male. A microscopically free resection margin was obtained in 83% of the patients. The median number of lymph nodes examined was 16 (range 2-40) with 58% of the patients having lymph node metastasis (n=14). Average lymph node ratio (positive nodes divided by total number of nodes found) was 0.12 with 4 patients (17%) having a lymph node ratio of ≥0.2. A standard pancreaticoduodenectomy was performed on 21 patients. The 3 remaining patients received a pylorus-preserving pancreaticoduodenectomy. One tumor was well differentiated (4%), 16 were moderately differentiated (67%) and 7 were poorly differentiated (29%). Most patients presented with a stage IIB tumor (n=14; 58%). The remaining tumors were staged IB (n=3; 13%) and IIA (n=7; 30%).

On the basis of these clinical characteristics, it can be concluded that the control group matched the study cohort. The control group consisted of 10 PDAC patients who had died within 6-18 months after resection (Table 2).

Genetic analysis of KRAS

Pyrosequencing for activating point mutations in KRAS was performed. Codon 12 and 13 were sequenced. One sample (4%) did not produce sufficient amplification and could therefore not be included. Two samples were KRAS wild type (9%). The most frequently observed mutation was $Kras^{G12D}$, which was identified in 12 cases (52%). Other mutations found were $Kras^{G12V}$ (n=5; 22%), $Kras^{G12C}$ (n=1; 4%) and $Kras^{G12R}$ (n=1; 4%). One case presented with two separate mutations, $Kras^{G12D}$ and $Kras^{G12V}$. In the control group a similar set of mutations was found.

		Study group			
		Long-term survival		**Short-term survival**	
		Mean (range)	**Count (%)**	**Mean (range)**	**Count (%)**
Age		63 (41-80)		62 (50-71)	
Tumor size		2.7 (1.3-6.0)		3.1 (2.0-5.0)	
Lymph node radio		0.13 (0-0.67)		0.10 (0-0.31)	
Lymph node ratio	**<0.2**		18 (78.3)		9 (90)
	≥0.2		5 (21.7)		1 (10)
Differentiation grade	**Poor**		7 (29.2)		4 (40)
	Moderate		15 (62.5)		6 (60
	Well		1 (4.2)		0
	Unknown		1 (4.2)		0
Stage	**IB**		3 (12.5)		0
	IIA		7 (29.2)		3 (30)
	IIB		14 (58.3)		7 (70)

Table 2 Clinical characteristics of the long- and short-term survivors.

MSI analysis:

To examine whether MSI was (partly) responsible for the extended survival in our study cohort, MSI-analysis was performed. In 22 of the 24 cases, MSI analysis produced reliable results. All assays were repeated several times. Only 1 of the 22 cases revealed a MSI marker (MSI-low, 5%). All other 21 cases were microsatellite stable with stability in all 5 mononucleotide markers. Because these results did not indicate an increase in MSI in the study cohort, MSI analysis in the control group was not performed.

Immunohistochemistry of p53, Smad4, hENT1 and dCK:

Immunohistochemical analysis of p53 expression showed strong nuclear labeling indicating a defective protein in 17 of the 24 long-term survivors (71%). The remaining 7 tumors (29%) showed normal p53 expression. Persistent Smad4 expression, a feature reported to affect prognosis favorably, was observed in 58% of the long term survival tumors (n=14).

Both hENT1 and dCK expression are claimed to influence prognosis of PDAC by enhancing the sensitivity of the cancer cells for gemcitabine. Not all PDAC patients received gemcitabine as adjuvant therapy because 12 tumors were resected before gemcitabine was introduced as standard first-line treatment after resection. Staining of hENT1 was strongly positive in 11 of 24 PDAC cases (46%) and weakly positive in another 9 cases (38%). Four cases showed no staining of hENT1 (17%). Of the 12 patients not treated with gemcitabine, 7 showed high hENT1 expression (58%), 3 showed low hENT1 expression (25%) and 2 did not show hENT1 expression (17%). Of the 12 patients that did receive gemcitabine treatment, 4 showed high hENT1 expression (33%), 6 showed low hENT1 expression (50%) and 2 did not show hENT1 expression (17%). A significant difference in hENT1 expression was not found between the patient group that was treated with gemcitabine and the patient group that did not receive gemcitabine as adjuvant therapy.

dCK expression was absent in 4 of the 24 cases (17%). Five patients showed weak expression (20%) and 15 showed strong expression of the dCK protein (66%). As reported earlier, there was a strong relation between age and dCK expression. Patients over 75 years showed a significantly lower expression of dCK (p=0.008) as compared to patients under 75 years of age. dCK was expressed in 80% of the cases treated with gemcitabine as compared to 84% of the non-treated cases. Surprisingly, there was a significant correlation between dCK expression and hENT1 expression (p=0.006; Table 3).

Because the proteins that were examined revealed a percentage of affected tumors in the very long-term survivor cohort comparable with previously reported percentages in the general PDAC patient population, immunohistochemical evaluation of the proteins in the short-term survival cohort was not performed.

	dCK staining		
hENT Staining	**Absent**	**Weak**	**High**
Absent	2	0	2
Weak	2	5	2
High	0	0	11

Table 3 Correlation of the immunohistochemical evaluation of hENT1 and dCK expression with significant correlation (P=0.005).

Discussion

Although subsets of PDAC patients surviving >5 years are reported regularly [6, 20], there is no consensus on the exact percentage of 5-year survival. The explanation may be that in the majority of the studies, 5-year survival is calculated using statistical methods that result in actuarial survival instead of actual survival, which has been proven to exaggerate the outcome [21]. It was found that from the 72.209 resections studied, only approximately 700 patients survived 5 years or more (<1%). Another explanation for the wide range in 5-year survival data is that

often the histological diagnosis is not confirmed. In 2005, Carpelan-Holstrom *et al* [7] performed histological re-evaluation of 5-year survivors and found a bonafide PDAC in only 38% of the cases. It was concluded that numbers of long-term survival patients were a grant exaggeration of the actual percentage because the patients reported to survive quite possibly did not suffer from PDAC. Other studies confirmed that re-evaluation of long-term survival samples show a considerably lower percentage of true long-term survivors of PDAC than previously assumed [22, 23].

On the basis of these considerations, it can be assumed that the lower estimations of survival percentages are more likely to be close to the actual survival data and it has even been debated whether there is such a thing as curing PDAC [6, 20]. In the present study, we tried to settle this debate and investigated whether there are patients who survive PDAC. The databases of three large hospitals were searched for ≥10 year survivors of PDAC and only 24 cases were discovered. Of the 26 patients found in the JHU database, only 14 (58%) turn out to have a bonafide PDAC which is consistent with the statement made by Carpelan-Holstrom *et al.* that reported long-term survivors of PDAC often never suffered from PDAC but from another prognostically more favorable disease [7]. Although our investigation confirms that survival of PDAC patients is probably much rarer than assumed, we hereby prove that survivors of PDAC do exist, possibly patients who can be considered cured.

Besides conformation of long-term survivors of PDAC, the aim of the present study was to map out the clinicalopathological features of a ≥10 year survival cohort and to evaluate whether these characteristics differed from the average PDAC patient. Remarkably, on the basis of tissue samples and clinical history of these patients, very few features can explain this prolonged survival. Compared to the general PDAC patient population, the ≥10 year survivors exhibited comparable percentages of lymph node metastasis, lymph node ratio or tumor stage at diagnosis, all factors that have been reported to affect prognosis [8, 13, 14, 24].
An important reason for poor prognosis of PDAC is the late diagnosis which often makes curative treatment impossible. Therefore, the very long-term survivors may have been diagnosed at an earlier stage than the average PDAC patient who underwent resection. This assumption was contradicted by our data. Most of the very long-term survivors had a tumor stage T3N1Mx and 58% had lymph node metastasis at resection. Even more remarkable was the fact that of the 24 patients, 4 did not have cancer-free resection margins. This is remarkable because a tumor-free resection margin is one of the strongest prognostic characteristics known to affect survival [9, 25].

The evolution from a normal pancreatic cell to a PDAC cell is considered to be caused by an accumulation of genetic alterations starting with an activating mutation in the *KRAS2* oncogene, which is found to be affected in >95% of PDAC cases. This mutation is followed by inactivation of one of the tumor suppressor

genes, specifically *CDKN2A* or *TP53.* Another tumor suppressor gene often affected in PDAC is *SMAD4* [26, 27]. Patients with preserved Smad4 signaling have a significantly longer survival than patients with loss of Smad4 expression [27-29].

In order to evaluate whether carcinogenesis in the very long-term survival cohort occurred in a similar manner as described above, we screened our study cohort for these most commonly observed mutations in PDAC. No difference in incidence or location of *KRAS2* mutations was found between the study cohort and the general PDAC population [30] or between the study cohort and the short-term survival cohort. From the 24 ≥10 year survivors 91% exhibited an activating mutation in codon 12 of *KRAS2* whereas *KRAS2* mutations were found in 95% of the general PDAC patient population.

The percentage of very long-term survivor tumors that showed affected expression of p53, the protein product of *TP53*, also did not differ from the numbers in the literature. Earlier studies show that in approximately 50-75% of the PDAC cases, expression of p53 is affected [31]. In the study cohort this number was 71%. When evaluating Smad4, 58% of the long-term survivor cohort showed persistent expression of the protein. The literature reports that 40-55% of the PDACs shows persistent expression of Smad4 [27]. Although the number of tumors with unaffected Smad4 expression was slightly higher in the long-term survivor group, this difference was not significant.

MSI has been related to a better prognosis in many malignancies among which PDAC [14, 32-34]. We argued that MSI may have played a role in prolonging survival time in the very long-term survival cohort but only 1 of the 24 PDAC samples showed MSI in 1 of the 5 mononucleotide markers which excluded that MSI played a role in prolonging survival.

hENT1 and dCK are proteins that play a role in the efficacy of gemcitabine treatment. hENT1 is the primary gatekeeper for intracellular uptake of gemcitabine and dCK phosphorylates gemcitabine eventually resulting in the activation of the drug. Studies have shown that high hENT1 expression is related to longer disease-free and overall survival [10]. High dCK expression was shown to be an independent and strong prognostic factor in patients suffering from PDAC [35]. However, we did not find a significantly higher percentage of hENT1-expressing cells in the long-term survivor cohort, nor could we confirm the previously found prognostic advantage of dCK expression. Because only 50% of the long-term survivors were treated with gemcitabine, it has to be realized that the prognostic advantage of high hENT1/dCK expression only applies to tumors treated with gemcitabine. Surprisingly, there was a significant relation between strong expression of dCK and strong hENT1 expression. To the best of our knowledge, this correlation has not been found before and it may be valuable to further

investigate this interaction as this could potentially lead to improvements in the effectivity of gemcitabine treatment.

A possibility not yet discussed is that the long-term survival was not related to factors associated with the tumor but was caused by characteristics of the host. It is well known that the malignant pancreatic cells in a tumor are surrounded by a large population of stromal cells and that these stromal cells play a role in the tumor environment. Also, immunological response to the tumor may have affected prognosis. Surgical resection of the tumor removes the bulk of the tumor cells but, as illustrated by the high percentage of recurrence, often malignant cells are left behind. Possibly, the hosts immune response to these cells can affect whether or not recurrence occurs. Future studies on these host-specific characteristics may hold great value for the improvement of our understanding of patient-specific prognosis and could possibly give an explanation for the exceptionally long survival time in some patients.

This study was the first step in characterizing a particular group of long-term survivors of PDAC. As far as we know, never before had a cohort consisting of only ≥10-year survivors of PDAC been constructed. This study cohort will be further explored to continue the search for an explanation for the very long-term survival.

Reference list

1. American Cancer Society, *Cancer Facts and Figures 2010* 2010. Atlanta, GA.
2. Hezel AF, Kimmelman AC, Stanger BZ, et al., *Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev*, 2006. **20**(10): p. 1218-49.
3. Schneider G, Siveke JT, Eckel F, et al., *Pancreatic cancer: basic and clinical aspects.* Gastroenterology, 2005. **128**(6): p. 1606-25.
4. David M, Lepage C, Jouve JL, et al., *Management and prognosis of pancreatic cancer over a 30-year period.* Br J Cancer, 2009. **101**(2): p. 215-8.
5. Schnelldorfer T, Ware AL, Sarr MG, et al., *Long-term survival after pancreatoduodenectomy for pancreatic adenocarcinoma: is cure possible?* Ann Surg, 2008. **247**(3): p. 456-62.
6. Ferrone CR, Brennan MF, Gonen M, et al., *Pancreatic adenocarcinoma: the actual 5-year survivors.* J Gastrointest Surg, 2008. **12**(4): p. 701-6.
7. Carpelan-Holmstrom M, Nordling S, Pukkala E, et al., *Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry.* Gut, 2005. **54**(3): p. 385-7.
8. Riediger H, Keck T, Wellner U, et al., *The lymph node ratio is the strongest prognostic factor after resection of pancreatic cancer.* J Gastrointest Surg, 2009. **13**(7): p. 1337-44.
9. Bhatti I, Peacock O, Awan AK, et al., *Lymph node ratio versus number of affected lymph nodes as predictors of survival for resected pancreatic adenocarcinoma.* World J Surg, 2010, 34(4):768-75.
10. Farrell JJ, Elsaleh H, Garcia M, et al., *Human equilibrative nucleoside transporter 1 levels predict response to gemcitabine in patients with pancreatic cancer.* Gastroenterology, 2009. **136**(1): p. 187-95.
11. Giovannetti E, Del Tacca M, Mey V, et al., *Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine.* Cancer Res, 2006. **66**(7): p. 3928-35.
12. Goggins M, Offerhaus GJ, Hilgers W, et al., *Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+.* Am J Pathol, 1998. **152**(6): p. 1501-7.
13. Pantano F, Baldi A, Santini D, et al., *MUC2 but not MUC5 expression correlates with prognosis in radically resected pancreatic cancer patients.* Cancer Biol Ther, 2009. **8**(11): p. 996-9.
14. Yamamoto H, Itoh F, Nakamura H, et al., *Genetic and clinical features of human pancreatic ductal adenocarcinomas with widespread microsatellite instability.* Cancer Res, 2001. **61**(7): p. 3139-44.
15. Marechal R, Mackey JR, Lai R, et al., *Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma*. Clin Cancer Res, 2009. **15**(8): p. 2913-9.
16. Neoptolemo JP, Stocken DD, Dunn JA, et al., *Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial.* Ann Surg, 2001. **234**(6): p. 758-68.

17. Tsiatis AC, Norris-Kirby A, Rich RG, et al., *Comparison of sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations. diagnostic and clinical implications.* J Mol Diagn, 2010. 12(4):425-32.
18. Bacher JW, Flanagan LA, Smalley RL, et al., *Development of a fluorescent multiplex assay for detection of MSI-High tumors.* Dis Markers, 2004. **20**(4-5): p. 237-50.
19. Murphy KM, Zhang S, Geiger T, et al., *Comparison of the microsatellite instability analysis system and the Bethesda panel for the determination of microsatellite instability in colorectal cancers.* J Mol Diagn, 2006. **8**(3): p. 305-11.
20. Ahmad NA, Lewis JD, Ginsberg GG, et al., *Long term survival after pancreatic resection for pancreatic adenocarcinoma*. Am J Gastroenterol, 2001. **96**(9): p. 2609-15.
21. Gudjonsson B, *Pancreatic cancer: survival, errors and evidence*. Eur J Gastroenterol Hepatol, 2009. **21**(12): p. 1379-82.
22. Jorgensen MT, Fenger C, Kloppel G, et al., *Long-term survivors among Danish patients after resection for ductal adenocarcinoma of the pancreas.* Scand J Gastroenterol, 2008. **43**(5): p. 581-3.
23. Connolly MM, Dawson PJ, Michelassi F, et al., *Survival in 1001 patients with carcinoma of the pancreas.* Ann Surg, 1987. **206**(3): p. 366-73.
24. Han SS, Jang JY, Kim SW, et al., *Analysis of long-term survivors after surgical resection for pancreatic cancer.* Pancreas, 2006. **32**(3): p. 271-5.
25. Barugola G, Partelli S, Marcucci S, et al., *Resectable pancreatic cancer: who really benefits from resection?* Ann Surg Oncol, 2009. **16**(12): p. 3316-22.
26. Hahn SA, Schutte M, Hoque, AT, et al., *DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1*. Science, 1996. **271**(5247): p. 350-3.
27. Blackford A, Serrano OK, Wolfgang CL, et al., *SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer*. Clin Cancer Res, 2009. **15**(14): p. 4674-9.
28. Tascilar M, Skinner HG, Rosty C, et al., *The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma.* Clin Cancer Res, 2001. **7**(12): p. 4115-21.
29. Iacobuzio-Donahue CA, Fu B, Yachida S, et al., *DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer.* J Clin Oncol, 2009. **27**(11): p. 1806-13.
30. Hruban RH, van Mansfeld AD, Offerhaus GJ, et al., *K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization.* Am J Pathol, 1993. **143**(2): p. 545-54.
31. Barton CM, Staddon SL, Hughes CM, et al., *Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer.* Br J Cancer, 1991. **64**(6): p. 1076-82.
32. Parc Y, Gueroult S, Mourra N, et al., *Prognostic significance of microsatellite instability determined by immunohistochemical staining of MSH2 and MLH1 in sporadic T3N0M0 colon cancer.* Gut, 2004. **53**(3): p. 371-5.
33. Beghelli S, de Manzoni G, Barbi S, et al., *Microsatellite instability in gastric cancer is associated with better prognosis in only stage II cancers.* Surgery, 2006. **139**(3): p. 347-56.
34. Nakata B, Wang YQ, Yashiro M, et al., *Prognostic value of microsatellite instability in resectable pancreatic cancer.* Clin Cancer Res, 2002. **8**(8): p. 2536-40.

35. Marechal R, Mackey JR, Lai R, et al., *Deoxycitidine kinase is associated with prolonged survival after adjuvant gemcitabine for resected pancreatic adenocarcinoma.* Cancer, 2010, 15;116(22):5200-6.

Chapter 7

Stromal galectin-1 expression is associated with long-term survival in resectable pancreatic ductal adenocarcinoma

Ru Chen, Sheng Pan, Niki A. Ottenhof, Roeland F. de Wilde, Christopher L. Wolfgang, Zhaoli Lane, Jane Post, Mary P. Bronner, Ralph Hruban, Anirban Maitra, Teresa A. Brentnall

Cancer Biology & Therapy 2012, in press

Abstract

The overall 5-year survival rate for pancreatic ductal adenocarcinoma (i.e. PDAC) is a dismal 5%, although patients that have undergone surgical resection have a somewhat better survival rate of up to 20%. Very long-term survivors of PDAC (defined as patients with ≥10-year survival following apparently curative resection), on the other hand, are considerably less frequent. The molecular characteristics of very long-term survivors (VLTS) are poorly understood, but might provide novel insights into prognostication for this disease. In this study, a panel of five VLTS and stage-matched short-term survivors (STS, defined as disease-specific mortality within 14 months of resection) were identified, and quantitative proteomics was applied to comparatively profile tumor tissues from both cohorts. Differentially expressed proteins were identified in cancers from VLTS *versus* STS patients. Specifically, the expression of galectin-1 was 2-fold lower in VLTS compared to STS tumors. Validation studies were performed by immunohistochemistry (IHC) in two additional cohorts of resected PDAC, including: 1) an independent cohort of VLTS and 2) a panel of sporadic PDAC with a considerable range of overall survival following surgery. Immunolabeling analysis confirmed that significantly lower expression of stromal galectin-1 was associated with VLTS (P=0.02) and also correlated with longer survival in sporadic, surgically-treated PDAC cases (hazard ratio = 4.9, P = 0.002). The results from this study provide new insights to better understand the role of galectin-1 in PDAC survival, and might be useful for rendering prognostic information, and developing more effective therapeutic strategies aimed at improving survival.

Introduction

Invasive pancreatic ductal adenocarcinoma (PDAC), is the fourth leading cause of cancer-related deaths in the USA, with over 37,000 individuals estimated to die from this disease in 2011 [1]. The overall 5-year survival rate of pancreatic cancer is approximately 5%, which is due in large part to the fact that the vast majority of patients (~80%) are diagnosed with surgically unresectable, locally advanced or distant metastatic disease [1]. Chemo- and/or radiation therapy are only marginally effective, increasing survival by only a few months for most patients [2]. The best reported median survival outcomes with multimodality regimens like FOLFIRINOX are still in the range of 12 months for advanced disease [3].

In contrast to advanced disease, the outcome for patients that undergo surgical resection is considerably better, with 5-year survival rates of up to 20% reported in numerous studies [4-13]. Clinicopathological features associated with improved survival at 5 years post resection include small tumor size (<2 cm), negative lymph node status, negative resection margin (i.e. R0), absence of angiolymphatic and perineural invasion, and low grade of differentiation. Relatively longer (i.e. ≥5-year) survival after surgery for PDAC has been increasing for the last two decades, largely due to centralization of surgery to high-volume (i.e. expert) centers [14, 15]. Moreover, several clinicopathological characteristics have been demonstrated to have significant affect on long-term survival, such as small tumor size (<2 cm), negative lymph node status, negative resection margin (i.e. R0) and differentiation grade [15]. In addition, the relative levels of expression of certain drug transporters responsible for gemcitabine influx, such as the human equilibrative nucleoside transporter-1 (hENT-1) have been implicated in improved survival in the adjuvant setting [16].

Although survival to five years would be considered a "cure" in some cancers, this is unfortunately not true in the case of PDAC, with additional attrition of survivors occurring between five and ten years post-surgery [5]. Thus, "very long-term survivors" (VLTS) of PDAC (defined as patients with ≥10-year survival following resection) are quite uncommon, and even their very existence has been questioned in some studies, following careful re-evaluation of histopathology in the corresponding tumors [17, 18]. The most common reason for misclassification has been the inclusion of either ampullary cancers, or invasive cancers arising in the setting of a cystic neoplasm of the pancreas (intraductal papillary mucinous neoplasm, or IPMN), both of which have significantly better prognosis than conventional PDAC [11, 19-21]. Nonetheless, there are a handful of published studies which have identified ≥10-year survivors of histologically validated PDAC, usually at a frequency less than 5% in most series [4, 5, 12, 22]. Of note, these cohorts of VLTS patients not only include those with small T1, node-negative (N0) tumors, but also patients with more aggressive features, including T2-T4 sized lesions, and lymph node involvement [5]. This would suggest that the basis of VLTS in PDAC extends beyond simply resecting node-negative localized cases, but might indicate an intrinsic molecular phenotype of such tumors.

The objective of this study was to perform proteomic profiling of histologically validated PDACs from VLTS compared to stage-matched short-term survivors (STS, defined as patients with disease specific mortality within 14 months post-resection), in order to identify molecular networks that might be contributing to improved survival in this otherwise lethal disease. Our experiments have identified several candidates as being associated with long survival, and in particular, low expression of stromal galectin-1, was further validated in two independent cohorts of PDAC patients. These studies provide some of the first insights into molecular mechanisms underlying prolonged survival in PDAC.

Materials and Methods

Specimens:
This study was approved by the Human Subjects Division of the University of Washington, the Internal Review Board of the Cleveland Clinic, and the Internal Review Board of the Johns Hopkins Medical Institutions. Very long-term survivors (VLTS) and short-term survivors (STS) were identified as survivors of PDAC ≥10 years and <14 months, respectively, following surgical resection. Before inclusion, the histology was reviewed for all identified VLTS and STS cases from Johns Hopkins by two experts in pancreatic pathology (RHH and AM), in order to confirm the diagnosis of PDAC and exclude the possibility of confounding lesions like ampullary cancers or IPMNs. Finally, five VLTS and five STS were matched by tumor stage and grade in order to perform unbiased proteomic analysis (the changing standards for adjuvant chemotherapy over the past 15 years precluded us from matching cases by type of treatment received) (Supplemental Table 1). For validation studies, two additional cohorts were formed of patients from two institutions (Johns Hopkins and Cleveland Clinic). The first cohort consisted of 22 VLTS and 10 stage-matched STS, all obtained from the Hopkins archives. The second validation cohort included 106 patients, including PDAC (n=43), primary chronic pancreatitis (n=30), and benign normal pancreatic controls (n=33), which were used to construct a tissue microarray (TMA), subsequent to verification of the underlying pathology by an expert (MPB). Of note, the latter validation cohort was selected from patients undergoing pancreatic surgery without knowledge of the cancer survivor-status.

Protein extraction and sample preparation:
Protein extraction from formalin fixed paraffin embedded (FFPE) tissue was performed as previously described [23]. Briefly, slides holding 15µm, non-H&E stained sections of paraffinized tissue were heated and deparaffinized. One hematoxylin and eosin (H&E) stained reference section was examined under the microscope to delineate the areas with highest neoplastic cellularity (including both PDAC epithelium and associated stroma), while excluding obvious areas of non-neoplastic pancreatic acinar tissues and inflammatory infiltrates. Thereafter, ten unstained and deparaffinized serial sections of 10µm thickness each were used to obtain the aforementioned delineated area of cancerous tissue by manual dissection using a surgical knife and needle tip. The dissected cancerous tissue

samples were collected and transferred into 300µl of solution with 70% 50mM ammonium bicarbonate and 30% acetonitrile. The lysates were then incubated at 90°C for 30 minutes followed by 60°C for 120 minutes to rehydrate the proteins and hydrolyze the crosslinks. The samples were sonicated for 2 minutes followed by 1 minute incubation on ice. The process was repeated two more times. The homogenized samples were incubated at 60°C for 1 hr, followed by a second sonication step.

The protein lysates were then digested with sequencing-grade trypsin (Promega, catalog V5113) with a 1:50 ratio at 37°C for 18 hours. The digested samples were centrifuged at 1500xg for 10 minutes and the supernatants were collected. Equal amounts of the digested pancreatic cancer samples from each group of the STS and VLTS patients were pooled together to generate the pooled short-term sample and the pooled long-term sample, respectively. The pooled samples were buffer-exchanged into iTRAQ (Applied Biosystems, catalog 4368879) dissolution buffer (0.5 M triethylammonium bicarbonate). The samples were reduced with 50 mM tris-(2 carboxyethyl)phosphine (TCEP) and blocked with 200 mM methyl methanethiosulfonate (MMTS). The short-term and long-term samples were labeled with iTRAQ reagents of 114 and 117, respectively, and then combined. The combined labeled sample was fractionated with a strong-cation-exchange (SCX) spin column into 3 fractions. The samples were then purified with C18 columns (UltraMicroSpin Column / Vydac C18 silica, The Nest Group, catalog SUM SS18V), dried down and stored in -20°C until mass spectrometric analysis.

Mass spectrometry and proteomics data analysis:
Fractionated peptide samples were separated with reverse phase LC and spotted on a MALDI plate using a NanoLC 2-Dimensional HPLC system coupled with a Probot Micro Fraction collector (LC Packing/Dionex). The effluent from the capillary column was mixed with the re-crystallized α-cyano-4-hydroxycinnamic acid matrix solution with 1:1 ratio in a mixing tee then deposited onto a stainless MALDI plate with a 675-format (45x15). The spotted samples were calibrated and analyzed by an ABI 4800 MALDI TOF/TOF tandem mass spectrometer (Applied Biosystems) with reflector positive ion mode. Both MS and MS/MS data were acquired with an Nd:YAG laser with 200Hz repetition rate. For MS analysis, 800–4000 m/z mass range was used with 1000 acquisitions per spectrum. A maximum of 20 precursors per spot with minimum signal/noise ratio of 20 were selected for data-dependent MS/MS analysis. The CID (collision induced dissociation) was performed using air as the collision gas, and 2500 acquisitions were accumulated for each MS/MS spectrum.

The MS/MS spectra were searched against the International Protein Index (IPI) human protein database (version 3.29) from the European Bioinformatics Institute using GPS software v3.6 (Applied Biosystems) running the Mascot search algorithm (Matrix Science) for peptide and protein identification. Data searches were performed with the following criteria: fixed modifications of iTRAQ labeling on the N terminus and lysine (144.10) and methyl methanethiosulfonate on cysteine (46.01); and differential modification of methionine oxidation (15.99). A 95%

confidence interval was used for peptide identification. The iTRAQ quantification was performed using GPS software.

Immunohistochemistry (IHC):
IHC was performed on standard serial sections of the paraffin-embedded PDAC tissue sections and microarrays. Briefly, prior to incubation with the primary antibody, sections were pre-treated using a Borg antigen retrieval solution (Biocare Medical, catalog BD1000 S-250, MM, G1) in a pressure cooker for 15 minutes at 125 °C, followed by cooling at room temperature for 15 min. Thereafter, primary antibody to galectin-1 polyclonal antibody (1:200 dilution; Fitzgerald Industries International, catalog 10R-G100a) was applied at 37 °C followed by application of a diaminobenzidine detection kit (Ventana Medical Systems, catalog 760-091). Labeling was performed on an ES automatic immunohisto-chemical stainer (Ventana Medical Systems, catalog N750-BMKU-FS). Results were evaluated independently by two experienced GI-pathologists (MBP and ZL) without knowledge of patient survival information. As further detailed in the results section, galectin-1 expression was essentially restricted to the stromal compartment, with no expression within epithelial cells. Thus, labeling was assessed for the stromal compartment only. The number of stromal cells expressing galectin-1 varied widely between pathologic diagnoses and was scored semi-quantitatively from 0 to 3+. Specifically, cases with labeling of less than 5%, 5-32%, 33-67%, and greater than 67% of stromal cells were scored as 0, 1+, 2+, and 3+, respectively.

Results

Identification of differentially expressed proteins in cancers from VLTS versus STS patients:
Stable isotope-labeled based quantitative proteomics was performed on tumor tissues to reveal proteins associated with VLTS in PDAC. Equal amounts of proteins extracted from PDAC tissue from 5 VLTS and 5 STS samples were pooled to create a very long-term and short-term survivor reference sample, respectively. The two pooled samples were labeled with iTRAQ stable isotope tags individually, then combined, subjected to subsequent fractionation and purification, followed by mass spectrometry analysis as described previously [24]. Quantitative proteomics based on a LC MALDI TOF/TOF analysis revealed 26 differentially expressed proteins at ≥1.5-fold: 11 were over-expressed, and 15 were under-expressed in VLTS (Table 1).

The differentially expressed proteins were entered into the online DAVID Bioinformatics database for functional annotation [25, 26]. Seven of the proteins are involved in defense response (AZU1, DEFA3 RNASE3, C1QC, KRT1, THBS1 and FN1), all of which, except KRT1, were under-expressed in tumors from the VLTS cohort. Four proteins (AZU1, LGALS1, TXNDC5, and THBS1) are involved in regulation of apoptosis, and all four were under-expressed in VLTS. Some of these differentially expressed proteins, namely galectin 1 (LGALS1), thrombospondin 1 (THBS1), and S100A4 have previously been reported to be

overexpressed in PDAC tissues, and their overexpression is known to correlate with an adverse prognosis [24, 27, 28]. Among these differentially expressed proteins, the expression of galectin-1 (LGALS1) was almost 2–fold higher in the STS pool compared to matched VLTS pool (Figure 1). Related to this observation, we have previously identified increased galectin-1 expression in the stroma of PanIN lesions and stroma of PDAC [24]. Previous studies have reported the association between galectin-1 upregulation and advanced histological grade, systemic metastases, and immune evasion in the tumor microenvironment [29-31]. Base on these observations, we chose to further investigate galectin-1 in the context of PDAC patient survival.

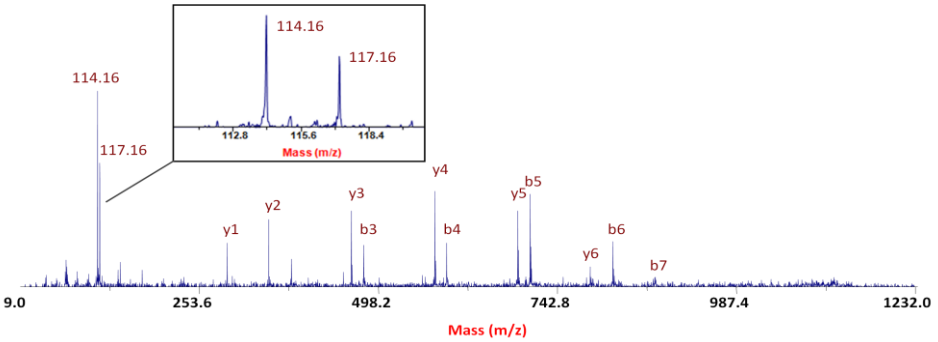


Figure 1 Identification of overexpression of galectin-1 in PDAC from STS in comparison to VLTS. The MS/MS spectrum leading to identification of a unique peptide (SFVLNLGK) from galectin-1 is displayed. The insert shows the iTRAQ reporting peaks of 114 and 117, representing STS and VLTS samples, respectively. The ratio of galectin-1 expression in STS relative to VLTS was calculated to be 1.91.

Correlation of galectin-1 with pancreatic cancer survival:

To verify the association of galectin-1 expression with survival and to localize the sub-compartment within which this expression was occurring, IHC was performed on an independent survivor validation cohort, including 22 VLTS and 10 STS (Figure 2). The expression of galectin-1 was essentially restricted to the peritumoral stromal compartment, with no expression within the neoplastic epithelium per se; therefore, only stromal labeling was evaluated. Using our previously described semi-quantitative scoring approach [32], galectin-1 immunolabeling was strong in the stromal cells in STS cancers, and had significantly less or minimal expression in VLTS samples (P = 0.02, Mann-Whitney U test). Eighty percent of STS showed strong immunolabeling (3+) for galectin-1 compared to 30% of VLTS. As seen in Figure 2D, ROC curve analysis to determine the performance of stromal galectin-1 immunolabeling in predicting very long-term survival showed an AUC of 0.75 (P = 0.026). Moreover, using negative or weak immunolabeling (IHC score ≤1) as cutoff, stromal galectin-1 achieved 64% sensitivity and 90% specificity in predicting very long-term survival.

To further validate the correlation of stromal galectin-1 expression with survival, a second independent validation set was used for galectin-1 IHC. After scoring of

the TMA (with blinding of survival data) of the 43 PDAC cases, 26 had confirmed survival time ranging from 2 to >72 months.

Protein Name	Gen Symbol	Protein ratio (VLTS/ STS)
Neutrophil defensin 3	DEFA3	0.38
Pancreatic prohormone	PPY	0.41
Galectin-1	LGALS1	0.52
Fibronectin	FN1	0.53
Eosinophil cationic protein (RNase 3)	RNASE3	0.53
Azurocidin (heparin binding protein)	AZU1	0.55
Laminin receptor-like protein	LAMR1P15	0.56
Thrombospondin-1	THBS1	0.56
L-lactate dehydrogenase A chain	LDHA	0.61
Protein S100-A4	S100A4	0.62
Glutathione S-transferase omega 1	GSTO1	0.62
Translocon-associated protein subunit alpha	SSR1	0.62
Complement C1q subcomponent subunit C	C1QC	0.66
cDNA FLJ52929, highly similar to Dolichyl-diphosphooligosaccharide--proteinglycosyltransferase 48 kDa subunit		0.66
thioredoxin domain containing 5	TXNDC5	0.67
Plastin-2	LCP1	1.50
Desmin	DES	1.51
chitinase-3-like protein 2	CHI3L2	1.55
Alpha-1 type I collagen	COL1A1	1.57
cDNA FLJ35635 fis, clone SPLEN2011805, highly similar to BONE/CARTILAGE PROTEOGLYCAN I (biglycan)	BGN	1.57
Keratin, type II cytoskeletal 1	KRT1	1.58
Glutathione peroxidase 3	GPX3	1.62
Full-length cDNA clone CS0DI085YI08 of Placenta of Homo sapiens		1.90
Prolargin (Proline-arginine-rich end leucine-rich repeat protein)	PRELP	2.31
Osteoglycin	OGN	3.09
Rheumatoid factor D5 light chain	V<kappa>3	17.92

Table 1 Differentially expressed proteins associated with very long-term survivor

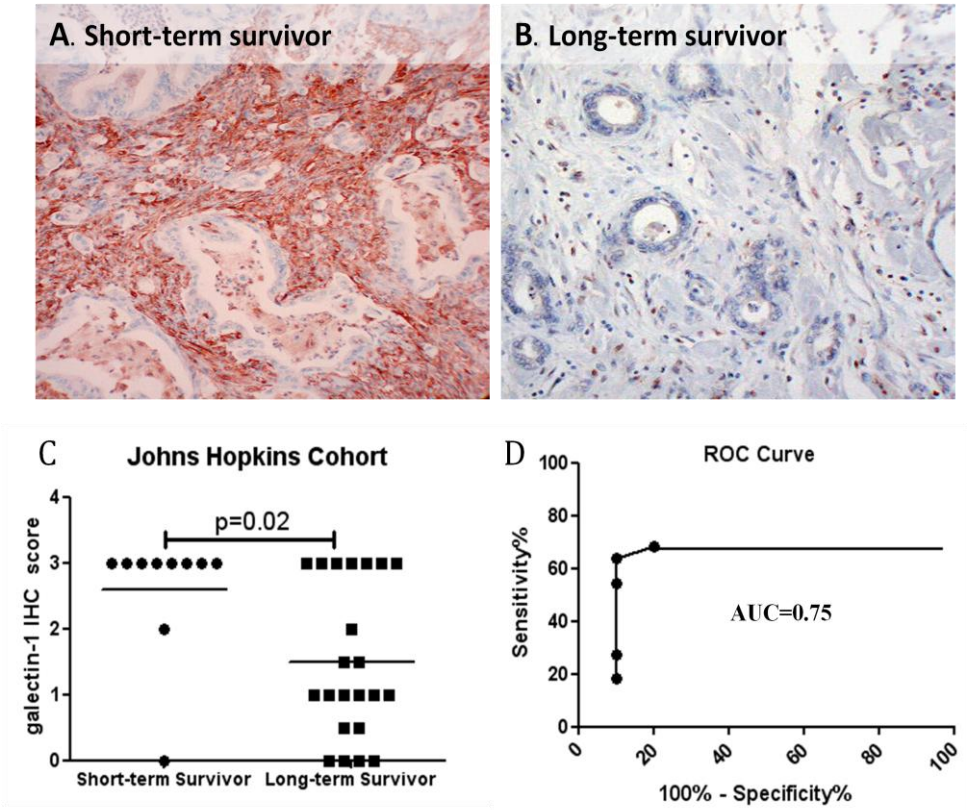


Figure 2 Galectin-1 staining in VLTS and STS in validation cohort 1 (Johns Hopkins cohort). Galectin-1 IHC of invasive pancreatic cancer displaying strong staining (3+, brown color) in desmoplatic stromal cells from a patient with short-term survival (A) and absent immunostaining in a VLTS patient (B). Distribution of galectin-1 staining from short-term survivors and very long-term survivors (C). ROC curve analysis displaying an ROC of 0.75, and 64% sensitivity and 90% specificity in predicting very long-term survival (D).

Survival curve analysis revealed that negative galectin-1 expression (IHC score 0) had significantly longer survival compared to cases with positive stromal galectin-1 labeling (IHC score ≥1+) (hazard ratio = 4.9, *P* = 0.002, Figure 3).

The median survival time was 24 months for galectin-1 positive cases, and 72 months for galectin-1 negative patients. Of interest, of the four cases that showed absent stromal galectin-1 immunolabeling, all had at least 72 months (6 years) survival post-resection.

Correlation of stromal galectin-1 expression with neoplastic progression:
Analysis of IHC revealed that galectin-1 expression was absent in all pancreatic epithelial cells. This was true for all three of the primary diagnostic categories, including normal pancreas, benign chronic pancreatitis and PDAC. However, the immunolabeling of stromal cells varied widely between underlying pathology (e.g.

normal pancreas versus pancreatitis versus PDAC), and dramatically increased with the development of neoplasia. There was virtually no or mild labeling (0-1+) in benign controls (n = 33), to limited labeling (1+) in primary chronic pancreatitis not associated with malignancy (n = 30), to the highest level (2+ to 3+) immunolabeling in stroma of PDAC (n=43) (Table 2 and Figure 4). Normal pancreas adjacent to PDAC displayed increased galectin-1 labeling compared to completely benign pancreatic tissue (Figure 4B vs. 4A). Secondary chronic pancreatitis (chronic pancreatitis associated with PDAC) also displayed increased galectin-1 labeling compared to the primary chronic pancreatitis. Intermediate labeling was observed in the stroma surrounding intermediate- and high-grade pancreatic intraepithelial neoplasia (PanIN-2 and PanIN-3, respectively).

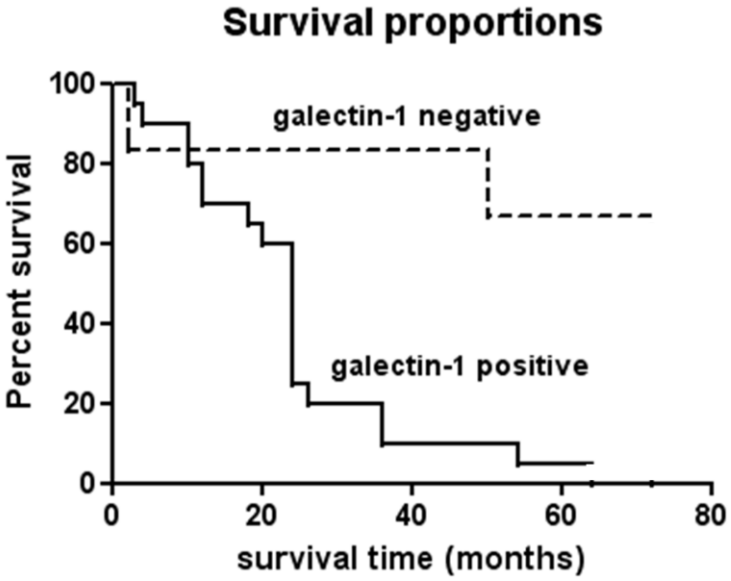


Figure 3 Survival analysis of galectin-1 immunostaining and patient survival in validation cohort 2 (Cleveland Clinic cohort). Patients with negative galectin-1 staining (IHC score 0) had significantly longer survival than the patients with positive galectin-1 staining (IHC score ≥1+) with hazard ratio 4.9 (*P* = 0.002).

Discussion

Very long-term survival (i.e. ≥10 years) in PDAC is extremely rare; it occurs only in very few patients after resection of their primary tumor. Several tumor characteristics have been identified to be of benefit to (very) long-term survival; however, its molecular characteristics remain unidentified. In this study, quantitative proteomics was applied to compare protein expression in PDAC tumor tissues in VLTS with stage -matched STS, followed by expression validation analysis using IHC.

Galectin-1, a member of beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions, was found to be underexpressed in the VLTS

pool, and its significance to survival was further studied by immunolabeling of tumor tissues using two independent patient cohorts with annotated survival data. First, galectin-1 expression was analyzed in surgical samples from 22 VLTS and 10 STS patients, confirming the significantly reduced stromal expression in VLTS. ROC curve analysis for galectin-1 expression as predictor of very-long term survival showed 64% sensitivity and 90% specificity. Second, galectin-1 immunolabeling using an existing TMA showed that PDAC patients with negative stromal galectin-1 staining had significantly longer survival than the patients with positive staining, with 4 of the PDAC patients with absent galectin-1 labeling alive at 72 months or greater post follow-up. These results suggest that increased/strong galectin-1 expression is a negative predictor of pancreatic cancer survival, or conversely, that absence or minimal stroma galectin-1 might be a harbinger of improved survival.

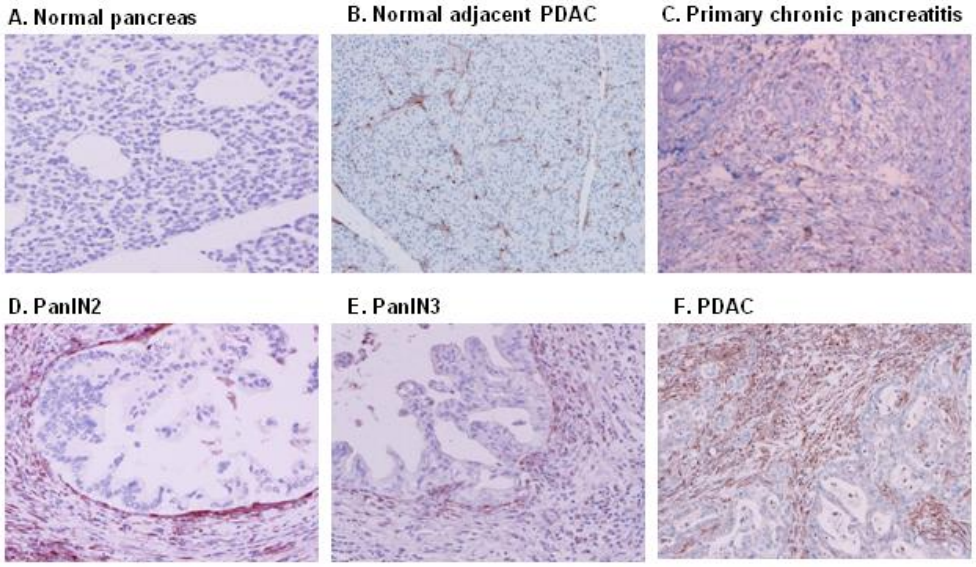


Figure 4 Representative galectin-1 staining of pancreas tissue microarrays. All ductal epithelial cells and neoplastic cells were negative for galectin-1 staining. The positive staining was detected only in the stromal cells for galectin-1 positive specimens. A. Normal pancreas (no staining); B. Normal pancreas adjacent to PDAC (1+); C. Primary benign chronic pancreatitis (1+); D. PanIN 2 (2+); E . PanIN3 (2+); F. PDAC (3+).

Galectin-1, protein product of the *LGASL1* gene, participates in diverse cell signaling pathways, including regulation of cell growth, cell migration, interaction with Ras signaling and modulation of innate and adaptive immune response [33]. Overexpression of galectin-1 has been well documented in many different tumor types, including breast adenocarcinoma, hepatocellular carcinoma, and oral squamous cell cancer, amongst others; and is generally localized to the stroma, and usually correlated with tumor aggressiveness and metastatic phenotypes [29-31]. Our current study provides evidence that expression of galectin-1 correlates negatively with survival; in line with earlier findings that overexpression is an adverse prognostic factor.

The mechanism in which galectin-1 acts to influence tumor properties remains to be elucidated for PDAC. A role has been suggested for galectin-1 in its contribution to tumor immune response escape through induction of apoptosis of activated T cells [34] and may provide an immunosuppressive environment at the tumor site in favor of tumor-immune escape [29]. More recently, galectin-1 has been shown to participate in angiogenesis; increased expression of galectin-1 was observed in the vasculature of several human malignant tumors [35]. More specifically, the uptake of galectin-1 by cultured endothelial cells promotes H-Ras signaling and stimulates endothelial cell proliferation and migration [35]. Conversely, knockdown of galectin-1 expression in endothelial cells was shown to inhibit proliferation and migration, and galectin-1-null mice displayed hampered tumor growth due to decreased angiogenic activity [36]. These results suggest the active role galectin-1 plays in the proliferation and migration of tumors via angiogenesis. Last, but perhaps most significant, galectin-1 is up-regulated in the initial stages of inflammation and wound repair signaling [37]. This is pertinent to cancer progression in that activated myofibroblasts are unable to partner with pancreatic cancer cells in assisting invasion and escape, unless a inflammatory trigger or wounding signal is present (*unpublished data*). This supports a paradigm of how some cancers can remain indolent, despite having the standard poor prognostic features such as poor differentiation, large size or more advanced staging. Galectin-1 may be one of the key inflammatory triggers that promote aggression and the lack of galectin-1 in the VLTS identifies the indolent tumor.

		IHC Score			
Diagnosis	**No.**	**0**	**1+**	**2+**	**3+**
Normal control pancreas	33	6 (18%)	27 (82%)	0	0
Normal adjacent to cancer	21	2 (10%)	15 (71%)	4 (19%)	0
Chronic pancretitis (primary benign)	30	2 (7%)	15 (50%)	10 (33%)	3 (10%)
Chronic pancreatitis (adjacent to cancer)	45	0	8 (18%)	25 (55%)	12 (27%)
Low grade dysplasia	14	1 (7%)	4 (29%)	5 (36%)	4 (29%)
High grade dysplasia	3	0	0	2 (67%)	1 (33%)
Pancreatic ductal adenocarcinoma	43	0	2 (5%)	9 (21%)	32 (74%)

Table 2. Galectin-1 staining in Pancreatic ductal adenocarcinoma TMA

Our finding that galectin-1 expression was observed in the stroma surrounding non-invasive precursor lesions of the pancreas, and increases in intensity with neoplastic progression suggests that it plays a role early in tumorigenesis and that there appears to be a “dose response”, as the stroma interacts with increasingly malignant cells. The finding of galectin-1 in the cancer-associated stroma, and not in the cancer epithelial cells, is particularly interesting in light of recent studies that identify stromal cells as key collaborators in cancer invasion and metastasis [38-41]. Galactin-1 was not present in stromal cells in normal pancreas and only marginally elevated in the stromal cells associated with pancreatitis. Targeted inhibition of galectin-1 expression has been proposed [36]. Anti-LGALS1 compounds are currently being investigated to target migrating cancer cells [33]. Rather than knocking-down the *LGALS1* gene in migrating tumor cells as has been previously suggested [36], it may be more effective inhibiting tumor invasion by blocking expression of *LGALS1* in the stromal cells. In such a setting it may be possible to turn an aggressive tumor into an indolent one.

In addition to galectin-1, our study identified several other aberrantly expressed proteins that could be further investigated in relation to pancreatic cancer survival. Two of the proteins overexpressed in the VLTS "pool" compared to the STS cohort by proteomic analysis were osteoglycin (OGN) (3-fold) and prolargin (PRELP) (2.3-fold), respectively. Osteoglycan belongs to the family of the small leucine-rich proteoglycans, that are involved in the regulation of cellular matrix and cellular growth. More specifically, the proteoglycan family are tissue organizers involved in orienting and ordering collagen fibrils during ontogeny and in pathological processes such as wound healing, tissue repair, and tumor stroma formation [42]. A recent study suggested that abundant expression of osteoglycan suppresses lymph node metastasis in highly metastatic hepatocellular cancer [43]. Overexpression of osteoglycan in VLTS might suggest that it suppresses metastasis in PDAC and/or regulates the tumor stroma to modulate tumor cell invasion. On a different note, prolargin (proline/arginine-rich end leucine-rich repeat protein) is a glycosaminoglycan and collagen-binding anchor protein that is abundantly expressed in cartilage, basement membranes, and developing bone [44]. Previous studies have suggested that prolargin acts as a cell-type specific inhibitor of the NFkB pathway that impairs osteoclastogenesis [44]. In addition, increased NFkB activity has been shown to promote angiogenesis, invasion, metastasis, as well as chemoresistance in pancreatic cancer [45]. Thus, in VLTS, increased prolargin could potentially inhibit or diminish the NFkB signaling and thereby blocking tumorigenesis, invasion, angiogenesis, and metastasis. Of note, comparable to galectin-1, these two overexpressed proteins are also localized to the stromal compartment, underscoring the critical role the microenvironment might be playing in modulating the natural history of PDAC. These potential targets will be investigated in future studies.

Our study has explored the protein expression pattern of very long-term survivors *versus* short-term survivors of surgically resected PDAC, and identified several aberrantly expressed candidates. In this study, the stromal galectin-1 was closely associated with pancreatic cancer survival, with a significantly decreased

expression observed in true VLTS survivors, as well as those at the higher end of the survival spectrum following resection. Its specific function and mechanism involved in PDAC survival warrants further investigation using functional studies in *bona fide* cancer associated fibroblasts (CAFs) and *in vivo* disease models. In addition to galectin-1, several other differentially expressed proteins were identified that could provide valuable insights into the molecular basis of prolonged survival in PDAC.

Reference list

1. Jemal A, Siegel R, Xu J, et al., *Cancer statistics, 2010.* CA Cancer J Clin 2010;60:277-300.
2. Stathis A, Moore MJ. *Advanced pancreatic carcinoma: current treatment and future challenges.* Nat Rev Clin Oncol 2010;7:163-172.
3. Conroy T, Desseigne F, Ychou M, et al., *FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer.* N Engl J Med 2011;364:1817-1825.
4. Adham M, Jaeck D, Le BJ, et al., *Long-term survival (5-20 years) after pancreatectomy for pancreatic ductal adenocarcinoma: a series of 30 patients collected from 3 institutions.* Pancreas 2008;37:352-357.
5. Bradley EL, III. *Long-term survival after pancreatoduodenectomy for ductal adenocarcinoma: the emperor has no clothes?* Pancreas 2008;37:349-351.
6. Cleary SP, Gryfe R, Guindi M, et al., *Prognostic factors in resected pancreatic adenocarcinoma: analysis of actual 5-year survivors.* J Am Coll Surg 2004;198:722-731.
7. Conlon KC, Klimstra DS, Brennan MF. L*ong-term survival after curative resection for pancreatic ductal adenocarcinoma. Clinicopathologic analysis of 5-year survivors.* Ann Surg 1996;223:273-279.
8. Han SS, Jang JY, Kim SW, et al., *Analysis of long-term survivors after surgical resection for pancreatic cancer.* Pancreas 2006;32:271-275.
9. Mosca F, Giulianotti PC, Balestracci T, et al., *Long-term survival in pancreatic cancer: pylorus-preserving versus Whipple pancreatoduodenectomy.* Surgery 1997;122:553-566.
10. Reddy S, Wolfgang CL, Cameron JL, et al., *Total pancreatectomy for pancreatic adenocarcinoma: evaluation of morbidity and long-term survival.* Ann Surg 2009;250:282-287.
11. Riall TS, Cameron JL, Lillemoe KD, et al., *Resected periampullary adenocarcinoma: 5-year survivors and their 6- to 10-year follow-up.* Surgery 2006;140:764-772.
12. Schnelldorfer T, Ware AL, Sarr MG, et al., *Long-term survival after pancreatoduodenectomy for pancreatic adenocarcinoma: is cure possible?* Ann Surg 2008;247:456-462.
13. Shimada K, Sakamoto Y, Nara S, et al., *Analysis of 5-year survivors after a macroscopic curative pancreatectomy for invasive ductal adenocarcinoma.* World J Surg 2010;34:1908-1915.
14. Finks JF, Osborne NH, Birkmeyer JD. *Trends in hospital volume and operative mortality for high-risk surgery.* N Engl J Med 2011;364:2128-2137.
15. Garcea G, Dennison AR, Pattenden CJ, et al., *Survival following curative resection for pancreatic ductal adenocarcinoma. A systematic review of the literature.* J Pancr 2008;9:99-132.
16. Kim R, Tan A, Lai KK, et al., *Prognostic roles of human equilibrative transporter 1 (hENT-1) and ribonucleoside reductase subunit M1 (RRM1) in resected pancreatic cancer*. Cancer 2011;117:3126-3134.
17. Carpelan-Holmstrom M, Nordling S, Pukkala E, et al., *Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry.* Gut 2005;54:385-387.
18. Jorgensen MT, Fenger C, Kloppel G, et al., *Long-term survivors among Danish patients after resection for ductal adenocarcinoma of the pancreas.* Scand J Gastroenterol 2008;43:581-583.

19. D'Angelica M, Brennan MF, Suriawinata AA, et al., *Intraductal papillary mucinous neoplasms of the pancreas: an analysis of clinicopathologic features and outcome.* Ann Surg 2004;239:400-408.
20. Maire F, Hammel P, Terris B, et al., *Prognosis of malignant intraductal papillary mucinous tumours of the pancreas after surgical resection. Comparison with pancreatic ductal adenocarcinoma.* Gut 2002;51:717-722.
21. Raimondo M, Tachibana I, Urrutia R, et al., *Invasive cancer and survival of intraductal papillary mucinous tumors of the pancreas.* Am J Gastroenterol 2002;97:2553-2558.
22. Allen PJ. *Pancreatic adenocarcinoma: putting a hump in survival.* J Am Coll Surg 2007;205:S76-S80.
23. Pan S, Chen R, Stevens T, et al., *Proteomics portrait of archival lesions of chronic pancreatitis*. PLoS ONE 2011;6:e27574.
24. Pan S, Chen R, Reimel BA, et al., *Quantitative proteomics investigation of pancreatic intraepithelial neoplasia.* Electrophoresis 2009;30:1132-1144.
25. Dennis G, Jr., Sherman BT, Hosack DA, et al., *DAVID: Database for annotation, visualization, and integrated discovery.* Genome Biol 2003;4:3.
26. Huang dW, Sherman BT, Lempicki RA. *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc 2009;4:44-57.
27. Chen R, Pan S, Brentnall TA, et al., *Proteomic profiling of pancreatic cancer for biomarker discovery.* Mol Cell Proteomics 2005;4:523-533.
28. Chen R, Brentnall TA, Pan S, et al., *Quantitative proteomics analysis reveals that proteins differentially expressed in chronic pancreatitis are also frequently involved in pancreatic cancer*. Mol Cell Proteomics 2007;6:1331-1342.
29. Daroqui CM, Ilarregui JM, Rubinstein N, et al., *Regulation of galectin-1 expression by transforming growth factor beta1 in metastatic mammary adenocarcinoma cells: implications for tumor-immune escape.* Cancer Immunol Immunother 2007;56:491-499.
30. Spano D, Russo R, Di MV, et al., *Galectin-1 and its involvement in hepatocellular carcinoma aggressiveness.* Mol Med 2010;16:102-115.
31. Zhong LP, Wei KJ, Yang X, et al., *Overexpression of galectin-1 is negatively correlated with pathologic differentiation grade in oral squamous cell carcinoma.* J Cancer Res Clin Oncol 2010;136:1527-1535.
32. Chen R, Yi EC, Donohoe S, et al., *Pancreatic cancer proteome: the proteins that underlie invasion, metastasis, and immunologic escape*. Gastroenterology 2005;129:1187-1197.
33. Camby I, Le MM, Lefranc F, et al., *Galectin-1: a small protein with major functions*. Glycobiology 2006;16:137R-157R.
34. Rubinstein N, Alvarez M, Zwirner NW, et al., *Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege.* Cancer Cell 2004;5:241-251.
35. Thijssen VL, Barkan B, Shoji H, et al., *Tumor cells secrete galectin-1 to enhance endothelial cell activity.* Cancer Res 2010;70:6216-6224.
36. Thijssen VL, Poirier F, Baum LG, et al., *Galectins in the tumor endothelium: opportunities for combined cancer therapy.* Blood 2007;110:2819-2827.
37. Gal P, Vasilenko T, Kostelnikova M, et al., *Open wound healing in vivo: monitoring binding and presence of adhesion/growth-regulatorygGalectins in rat skin during the course of complete re-epithelialization.* Acta Histochem Cytochem 2011;44:191-199.
38. Bhowmick NA, Neilson EG, Moses HL. *Stromal fibroblasts in cancer initiation and progression.* Nature 2004;432:332-337.
39. Chu GC, Kimmelman AC, Hezel AF, et al., *Stromal biology of pancreatic cancer.* J Cell Biochem 2007;101:887-907.

40. De Wever O, Demetter P, Mareel M, et al., *Stromal myofibroblasts are drivers of invasive cancer growth.* Int J Cancer 2008;123:2229-2238.
41. De Wever O, Mareel M. *Role of tissue stroma in cancer cell invasion.* J Pathol 2003;200:429-447.
42. Iozzo RV. *The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth.* Crit Rev Biochem Mol Biol 1997;32:141-174.
43. Cui X, Song B, Hou L, et al., *High expression of osteoglycin decreases the metastatic capability of mouse hepatocarcinoma Hca-F cells to lymph nodes.* Acta Biochim Biophys Sin (Shanghai) 2008;40:349-355.
44. Rucci N, Rufo A, Alamanou M, et al., *The glycosaminoglycan-binding domain of PRELP acts as a cell type-specific NF-kappaB inhibitor that impairs osteoclastogenesis.* J Cell Biol 2009;187:669-683.
45. Holcomb B, Yip-Schneider M, Schmidt CM. *The role of nuclear factor kappaB in pancreatic cancer and the clinical applications of targeted therapy.* Pancreas 2008;36:225-235.

Chapter 8

Notch-1 inhibition in pancreatic ductal adenocarcinoma with a receptor-specific blocking antibody diminishes anchorage-independent growth but not growth rate

Niki A. Ottenhof, Sonal Gupta, Masamichi Mizuma, Cornelis J.F. van Noorden, G. Johan A. Offerhaus, Anirban Maitra

Manuscript in preparation

Abstract

Background: Upregulation of the embryonic Notch signaling pathway has been observed in several different malignancies among which pancreatic ductal adenocarcinoma (PDAC). Because specific antibodies against the different components of the Notch signaling pathway were not available in earlier reports, less specific methods were used to suppress Notch signaling. Recently, a Notch1-receptor blocking antibody (N1-AB) was developed and made available for solid tumor research.

Methods:. The effectiveness and specificity of N1-AB was determined using qRT-PCR to detect expression of different Notch target genes. Two PDAC cell lines were selected for testing based on measurement of Notch-pathway component expression by qRT-PCR. To establish the effects of inhibition of Notch signaling on growth rate and colony forming potential, cells were treated with N1-AB. Various functional assays were performed, including cell viability and anchorage-independent growth assays. In order to compare the current study and earlier reports, a cell viability assay was also conducted using a γ-secretase inhibitor (GSI), a component of the Notch pathway that is vital for signaling.

Results: Treatment with N1-AB did not affect cell viability or growth rate of cells irrespective of its concentration. However, cell viability was diminished in cells that were treated with the GSI. Colony-forming potential was consistently diminished in the N1-AB-treated cells, revealing a 2-fold diminished number of colonies in anchorage-independent growth assays.

Conclusions: Our results indicate that Notch signaling does not affect growth rate of PDAC cells. This finding is in conflict with previous reports. Because GSI diminished growth rate of PDAC cells in our study, it suggests that the results from earlier studies may have been caused by non-specific interference of treatment. Notch signaling diminished anchorage-independent growth which suggests that the Notch pathway may play a role in migration and invasion of PDAC cells.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most dismal malignancies with a 5-year survival rate of less than 5% and a median survival of only 6 months. Despite recent advances made in the understanding of pancreatic carcinogenesis, these numbers have only slightly changed over the last 2 decades and pancreatic cancer is still responsible for the fourth largest number of cancer-related deaths in the USA [14]. Surgical resection remains the only curative treatment but since most patients present with locally advanced or distant metastatic disease, only 15-20% of patients qualify for treatment [14]. Currently, gemcitabine is one of the few adjuvant therapies proven to increase survival although this effect is marginal [4].

It is well established that PDAC develops through a stepwise progression from precursor lesions called pancreatic intraepithelial neoplasia (PanIN) to invasive malignancies [11, 13]. Besides multiple high-incidence mutations, re-activation of embryonic signaling pathways has been linked to tumorigenesis in PDAC. One of these embryonic signaling pathways, the Notch pathway, initially received attention in 1991 when Ellisen *et al* [8] described an activating mutation in the *NOTCH1* gene as cause of human T-cell acute lymphoblastic leukemia (T-cell ALL). In addition to human T-cell ALL, an oncogenic role for the Notch signaling pathway has been described in non-small cell lung cancer [6], ovarian carcinomas [21] and also PDAC [18].

The Notch pathway plays an important role in pancreatic organogenesis but after the pancreas has been formed, the signaling pathway returns to a dormant stage, only to be re-activated upon tissue damage [2, 23]. Several studies have shown up-regulation of Notch pathway activity in PDAC and this pathway is therefore a potential new target for treatment [17-19]. Moreover, a recent study reported that active Notch signaling can synergize with mutational *KRAS2* expression in initiation of PanIN development and invasive carcinoma in the mouse pancreas [7]. Given the high incidence of mutational expression of *KRAS2* in PDAC (>95%), Notch might therefore play an important role in disease initiation and progression.

Indications have been found that a subset of the cells in a tumor, the tumor-initiating cells (TICs), posses tumor-initiating potential with unlimited clonogenicity, increased migration and high resistance against chemotherapy [3]. Active Notch signaling has been linked specifically to these TICs and a better understanding of the exact role of Notch signaling in PDAC development could therefore be critical [1, 12].

Currently, 4 different *NOTCH* genes (*NOTCH1-4*) are known, each coding for a transmembrane receptor. Upon binding of a Notch-ligand (Jagged1,2 and DLL1,3,4) tumor necrosis factor alpha-converting enzyme (TACE), a metalloprotease of the ADAM family, performs the first cleavage of the Notch receptor which is immediately followed by a second cleavage by the γ-secretase complex. This last cleavage results in the release of the Notch intracellular domain (NICD). The NICD then migrates to the nucleus where it interacts with various DNA binding proteins leading to transcription of the Notch-target genes (Figure 1)(20). Transcriptional activity of Notch signaling leads to a wide range of cellular processes including decreased apoptosis, escape from cell cycle arrest and

decreased differentiation which are all features of carcinogenesis [18]. Notch-target genes are numerous and include members of the Hes and Hey families of genes [20].

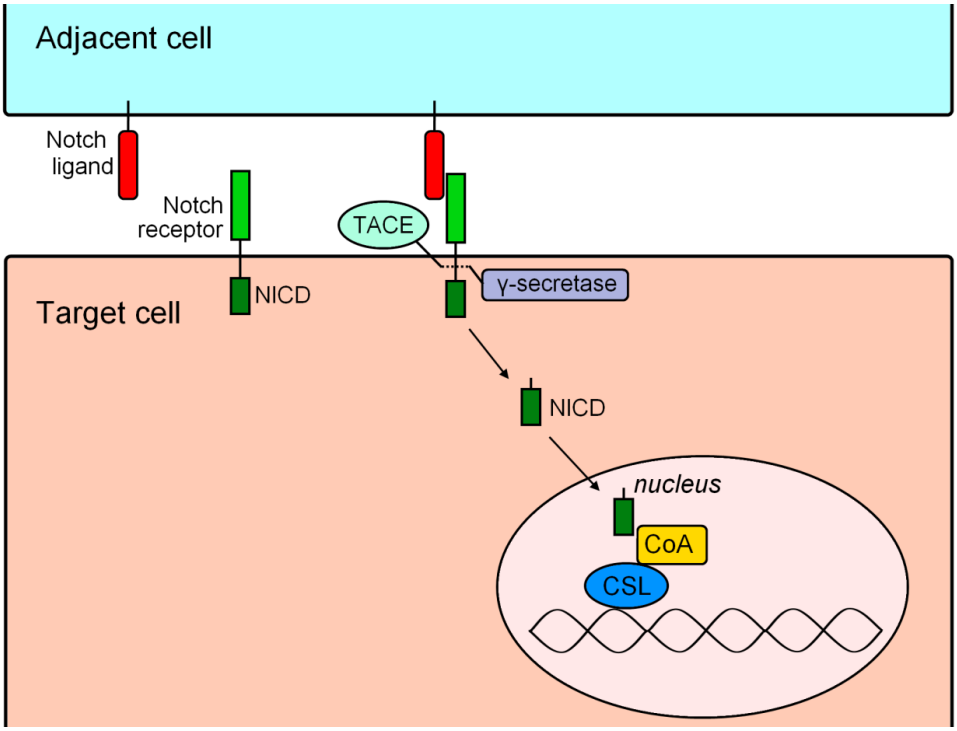


Figure 1 The Notch pathway. When a ligand, located on an adjacent cell, binds with one of the Notch receptors, the tumor necrosis factor alpha-converting enzyme and the γ-secretase complex cleave off the Notch intracellular domain. This domain migrates to the nucleus where it collaborates with DNA-binding proteins as transcription factor. TACE: tumor necrosis factor alpha-converting enzyme; NICD: Notch intracellular domain; CSL and CoA: DNA binding proteins.

Because a receptor-specific antibody against Notch receptors was not available, earlier reported studies used γ-secretase inhibitors (GSIs) or receptor-specific siRNAs to block Notch signaling. These types of treatment resulted in decreased proliferation, decreased anchorage-independent growth, increased apoptosis and decreased invasive growth in PDAC cells [19, 22, 25, 26].
Recently, Yagita *et al* [27] produced a series of monoclonal antibodies designed to block Notch signaling by inhibiting receptor-specific ligand binding. In the present study, we attempt to specifically identify the effect of Notch1 inhibition in PDAC using the Notch1-receptor blocking antibody (N1-AB). Various PDAC cell lines were treated with N1-AB and its effects on growth rate and anchorage-independent growth of the cells were evaluated. In order to compare the results with previously reported data, a cell viability assay after treatment with a GSI was performed as well.

Materials and methods

PDAC cell lines

Eleven PDAC cell lines (PANC-1, Capan-1, JD13D, E3, Panc8.13, Panc198, Pancc2.5, Panc3,014, Panc5.4, A38.5 and Panc10.05) were grown as previously described [5]. Immortalized nonmalignant human pancreatic epithelial cells (hTERT-HPNE) were cultured as described earlier [15] and used for normalization of Notch-pathway expression levels.

Activity of the Notch pathway was evaluated using quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the Notch receptor genes (*NOTCH1-4*), ligands (DLL1,2-4 and Jagged 1-2), and the Notch-target genes *HES1* and *HEYL*. RNA was isolated from quick frozen pallets of cells using the RNAeasy mini kit (Qiagen, Valencia CA, USA) according to the protocol provided by the manufacturer. With the use of the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad CA, USA), cDNA was constructed. Reactions were run on the StepOnePlus™ real-time PCR system (Applied Biosystems, Carlsbad CA, USA). Each reaction vial contained 10 µl of Fast Syber Green (Applied Biosystems), 1 µl of each primer (10 mM), 1 µg of cDNA and 7µl of DEPC-treated water. Primers were designed using Primer3Plus and blasted for cross reaction (sequences available upon request). Cycling conditions were as follows: 95°C for 15 min, 42 cycles of 95°C for 20 sec, 60°C for 30 sec, 72°C for 10 sec. The reaction was terminated at 4°C.

Antibody effectivity

To asses the effects of N1-AB treatment on Notch signaling, E3 and JD13D cells were incubated in the presence or absence of N1-AB (50 µg/ml) for 24 hours. Afterwards, the cells were collected and immediately frozen. RNA was isolated and cDNA constructed after which qRT-PCR for the Notch target genes *HES1* and *HEYL* was performed as described above.

GSI-18 synthesis

Synthesis of the GSI [11-endo]-*N*-(5,6,7,8,9,10-hexahydro-6,9-methanobenzo[a] [8]annulen-11-yl)-thiophene-2-sulfonamide (also known as GSI-18) has been described earlier [16, 19]. GSI-18 was used as treatment in the cell viability assay as described below.

Cell viability assay

Growth inhibition was assessed as described earlier [19]. Briefly, 2,000 cells per well were plated in 96-well plates and treated with 0, 50 and 100 µg/ml N1-AB or 0, 10, 20, 40 and 100 µmol/L of GSI-18 for 72 h. Relative growth inhibition was measured using the CellTiter 96 reagent (Promega, Madison WI, USA), as described in the manufacturer's protocol. Light intensity was measured with the fluorometric multitask plate counter Wallac 1420 Victor 2 (Beckman Coulter, Brea CA, USA). Blank wells were included to eliminate background luminescence.

Anchorage-independent growth

Anchorage-independent growth was assessed as previously described [19]. Briefly, soft agar assays were set up in 6-well plates, each well containing a bottom layer of 1% agarose (Invitrogen) in serum free DMEM, a middle layer of 0.6% agarose including 10,000 cells in serum free DMEM, and a top layer of serum free DMEM only. Before plating, cells were incubated for 30 minutes in the presence or absence of 50 µg/ml N1-AB. After 21 days, the upper layer was removed and colonies were visualized by staining with crystal violet. Colony formation was assessed using the Biorad ChemiDoc XRS and the colony counting function of the Quantity One software (Biorad Laboratories, Hercules CA, USA).

Statistical analysis

Two-tailed *t* tests were performed using GraphPad Prism for Windows version 5 (GraphPad software, La Jolla CA, USA). $P < 0.05$ was regarded as statistically significant. Results in bar diagrams are plotted as means +/- SDs if not otherwise indicated.

Results

PDAC cell lines

Two cell lines, JD13D and E3, of the 11 PDAC cell lines tested, showed the most active signaling (results not shown) and were selected for all further experiments.

Antibody effectivity

Treatment with N1-AB resulted in reduced Notch target expression. Expression of both *HES1* ($p<0.001$) and *HEYL* ($p<0.001$) was significantly diminished after treatment. Residual expression of both *HES1* and *HEYL* was observed after treatment indicating that Notch signaling was not completely abolished (Figure 2). Inhibition of expression was observed in both JD13D and E3 cells.

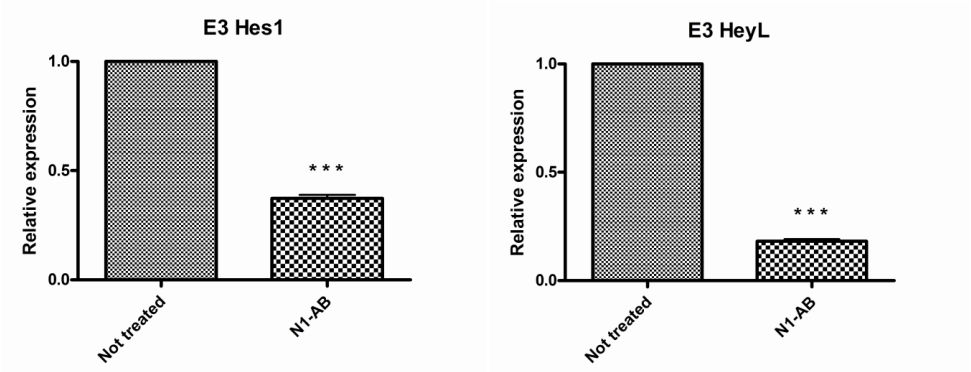


Figure 2 qRT-PCR data for Notch targets showing significant downregulation of expression of both Hes1 and HeyL upon N1-AB treatment in the PDAC cell line E3. ***p<0.001.

Cell viability

To examine the effect of Notch-1 inhibition on the growth rate of cells and to exclude possible toxicity of N1-AB, a cell viability assay was conducted. As depicted in Figure 3, treatment with N1-AB did not affect the number of viable cells. However, treatment with GSI-18 significantly reduced growth rate in a concentration-dependent manner in both cell lines.

Anchorage-independent growth

Anchorage-independent growth was reduced upon treatment with the N1-AB. Consistently, a 2-fold reduction in number of colonies was observed in N1-AB-treated JD13D and E3 cells although for E3 this decline was not significant (p=0.06) (Figure 4).

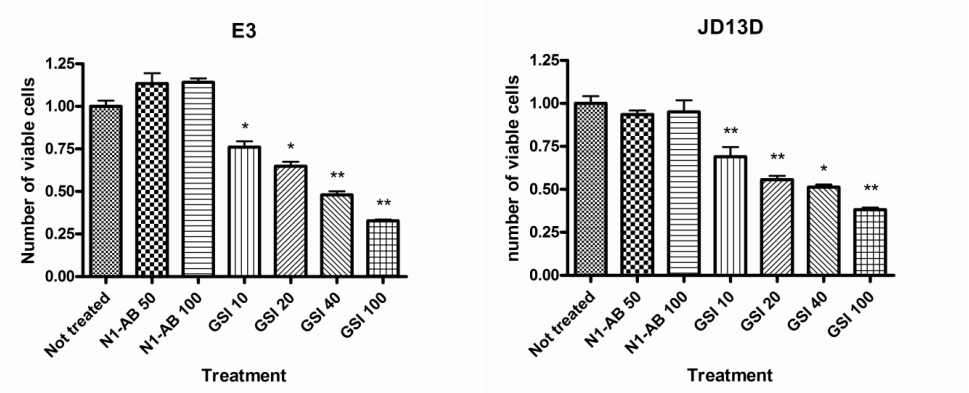


Figure 3 Cell viability assay shows no difference in number of viable cells when comparing untreated cells to N1-AB treated cells. Treatment with GSI-18 has a significant concentration-dependent inhibitory effect on the number of viable cells. *p<0.05, **p<0.01.

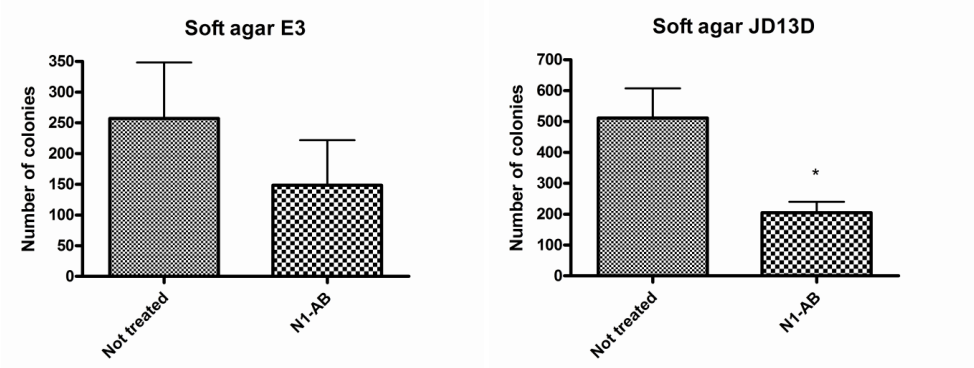


Figure 4 Soft agar assays of E3 (p=0.06) and JD13D (p=0.04) cells show a 2-fold decline in number of colonies after N1-AB treatment. *p<0.05.

Discussion

A role of the Notch signaling pathway in PDAC has been reported in earlier studies. Inhibition or activation of the Notch pathway indicated that specifically the Notch-1 receptor should be considered as a new target for therapy [7, 18, 19, 22, 26]. Multiple anti-tumor effects were reported including inhibition of growth rate, decreased anchorage-independent growth and decreased tumor growth *in vivo* [19, 26]. However, these studies used either GSIs or siRNAs and both have been shown to interact with other compounds than their initial targets. Han *et al* [10] found that the observed effects of γ-secretase inhibition in breast cancer were not caused by Notch inhibition but by proteasome inhibition. Moreover, Tasaka et al [24] observed a role for the γ–secretase complex in the response to taxane, inducing apoptosis in PDAC cells, whereas these effects were not found upon Notch inhibition. Therefore, it may be possible that the previously reported effects of GSI treatment of PDAC were not caused by Notch inhibition alone. In contrast to the methods previously used, we are the first to use a receptor-specific antibody against one of the Notch pathway components in solid tumor research.

The results of the present study indicate that Notch signaling does not affect the growth rate of PDAC cells. Cell viability was unaffected by N1-AB treatment whereas qRT-PCR showed distinct downregulation of Notch signaling upon N1-AB treatment. For this study, only the N1-AB was used and it can therefore be argued that cell viability and apoptosis are regulated by other components in the Notch pathway. However, this is unlikely because as far as we know, activation of each Notch receptor leads to transcription of the same target genes irrespective of the type of receptor [20]. It can not be disputed however, that the different components of the Notch pathway possibly have unique functions in cells that are currently unknown.

The lack of effect of N1-AB on cell viability is in conflict with previous studies. Therefore, we tested whether these findings were caused by differences in cell lines or methods used and thus a GSI-treatment group was included. Treatment with GSI-18 downregulated growth rate in a concentration-dependent manner. On the basis of these findings, we like to conclude that the effects observed in previous studies were not necessarily caused by inhibition of the Notch pathway.
Anchorage-independent growth was affected by treatment with the N1-AB. Consistently, a 2-fold decline in number of colonies was found in the treatment groups of both E3 and JD13D cells. As anchorage-independent growth is one of the major features for tumor spread, this result indicates that PDAC tumor spread rather than tumor size is affected by Notch signaling. However, the effect of Notch-1 signaling on anchorage-independent growth was much less pronounced than previously reported [19, 26]. Because earlier studies also found differences in cell viability and growth rate it is not possible to compare these results to our findings as cell viability also affects the anchorage-independent growth assay.

As far as we know, this study is the first to describe inhibition of Notch-signaling in PDAC using a receptor-specific monoclonal antibody. Inhibition does not affect

proliferation but rather it reduces migration and invasion of PDAC cells. Further research is needed to elucidate whether Notch receptor-specific blockade has potential as treatment of PDAC.

Reference list

1. Almoguera C, Shibata D, Forrester K, et al., *Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes.* Cell. 1988;53:549-554.
2. Apelqvist A, Li H, Sommer L, et al., *Notch signalling controls pancreatic cell differentiation.* Nature. 1999;400:877-881.
3. Bao B, Wang Z, Ali S, et al., *Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells.* Cancer Lett, 2011.307:26-36.
4. Burris HA, 3rd, Moore MJ, Andersen J, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* J Clin Oncol. 1997;15:2403-2413.
5. Calhoun ES, Hucl T, Gallmeier E, et al., *Identifying allelic loss and homozygous deletions in pancreatic cancer without matched normals using high-density single-nucleotide polymorphism arrays.* Cancer Res. 2006;66:7920-7928.
6. Dang TP, Gazdar AF, Virmani AK, et al., *Chromosome 19 translocation, overexpression of Notch3, and human lung cancer.* J Natl Cancer Inst. 2000;92:1355-1357.
7. De La O J, Murtaugh LC, *Notch and Kras in pancreatic cancer: at the crossroads of mutation, differentiation and signaling.* Cell Cycle. 2009;8:1860-1864.
8. Ellisen LW, Bird J, West DC, et al., T*AN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms.* Cell. 1991;66:649-661.
9. Geer RJ, Brennan MF, *Prognostic indicators for survival after resection of pancreatic adenocarcinoma.* Am J Surg. 1993;165:68-72; discussion 72-63.
10. Han J, Ma I, Hendzel MJ, et al., *The cytotoxicity of gamma-secretase inhibitor I to breast cancer cells is mediated by proteasome inhibition, not by gamma-secretase inhibition.* Breast Cancer Res. 2009;11:R57.
11. Hruban RH, Adsay NV, Albores-Saavedra J, et al., *Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions.* Am J Surg Pathol. 2001;25:579-586.
12. Hruban RH, van Mansfeld AD, Offerhaus GJ, et al., *K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization.* Am J Pathol. 1993;143:545-554.
13. Koorstra JB, Hustinx SR, Offerhaus GJ, et al., *Pancreatic carcinogenesis.* Pancreatology. 2008;8:110-125.
14. Koorstra JB, Karikari CA, Feldmann G, et al., *The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target.* Cancer Biol Ther. 2009;8:618-626.
15. Lawson T, Ouellette M, Kolar C, et al., *Culture and immortalization of pancreatic ductal epithelial cells.* Methods Mol Med. 2005;103:113-122.
16. Lewis SJ, Smith AL, Neduvelil JG, et al., *A novel series of potent gamma-secretase inhibitors based on a benzobicyclo[4.2.1]nonane core.* Bioorg Med Chem Lett. 2005;15:373-378.
17. Maitra A, Hruban RH, *Pancreatic cancer.* Annu Rev Pathol. 2008;3:157-188.
18. Miyamoto Y, Maitra A, Ghosh B, et al., *Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis.* Cancer Cell. 2003;3:565-576.
19. Mullendore ME, Koorstra JB, Li YM, et al., *Ligand-dependent Notch signaling is involved in tumor initiation and tumor maintenance in pancreatic cancer.* Clin Cancer Res. 2009;15:2291-2301.

20. Mumm JS, Kopan R, *Notch signaling: from the outside in.* Dev Biol. 2000;228:151-165.
21. Park JT, Li M, Nakayama K, et al. *Notch3 gene amplification in ovarian cancer.* Cancer Res. 2006;66:6312-6318.
22. Plentz R, Park JS, Rhim AD, et al. *Inhibition of gamma-secretase activity inhibits tumor progression in a mouse model of pancreatic ductal adenocarcinoma.* Gastroenterology. 2009;136:1741-1749 e1746.
23. Siveke JT, Lubeseder-Martellato C, Lee M, et al. *Notch signaling is required for exocrine regeneration after acute pancreatitis.* Gastroenterology. 2008;134:544-555.
24. Tasaka T, Akiyoshi T, Yamaguchi K, et al. *Gamma-secretase complexes regulate the responses of human pancreatic ductal adenocarcinoma cells to taxanes.* Anticancer Res. 2010;30:4999-5010.
25. Wang Z, Azmi AS, Ahmad A, et al. *TW-37, a small-molecule inhibitor of Bcl-2, inhibits cell growth and induces apoptosis in pancreatic cancer: involvement of Notch-1 signaling pathway.* Cancer Res. 2009;69:2757-2765.
26. Wang Z, Zhang Y, Li Y, et al. *Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells.* Mol Cancer Ther. 2006;5:483-493.
27. Yamanda S, Ebihara S, Asada M, et al. *Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade.* Blood. 2009;113:3631-36.

Chapter 9

MK-1775, a potent Wee1 inhibitior, synergizes with gemcitabine to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts

N. V. Rajeshkumar, Elizabeth De Oliveira, Niki A. Ottenhof, James Watters, David Brooks, Tim Demuth, Stuart D. Shumway, Shinji Mizuarai, Hiroshi Hirai, Anirban Maitra, Manuel Hidalgo

Clinical Cancer Research 2011; 17: 2799-2806

Abstract

Purpose: Investigate the efficacy and pharmacodynamic effects of MK-1775, a potent Wee1 inhibitor, in both monotherapy and in combination with gemcitabine (GEM) using a panel of p53-deficient and p53 wild-type human pancreatic cancer xenografts.

Experimental Design: Nine individual patient-derived pancreatic cancer xenografts (6 with p53-deficient and 3 with p53 wild-type status) from the PancXenoBank collection at Johns Hopkins were treated with MK-1775, GEM, or GEM followed 24 hour later by MK-1775, for 4 weeks. Tumor growth rate/regressions were calculated on day 28. Target modulation was assessed by Western blotting and immunohistochemistry.

Results: MK-1775 treatment led to the inhibition of Wee1 kinase and reduced inhibitory phosphorylation of its substrate Cdc2. MK-1775, when dosed with GEM, abrogated the checkpoint arrest to promote mitotic entry and facilitated tumor cell death as compared to control and GEM-treated tumors. MK-1775 monotherapy did not induce tumor regressions. However, the combination of GEM with MK-1775 produced robust antitumor activity and remarkably enhanced tumor regression response (4.01-fold) compared to GEM treatment in p53-deficient tumors. Tumor regrowth curves plotted after the drug treatment period suggest that the effect of the combination therapy is longer-lasting than that of GEM. None of the agents produced tumor regressions in p53 wild-type xenografts.

Conclusions: These results indicate that MK-1775 selectively synergizes with GEM to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts.

Introduction

Pancreatic ductal adenocarcinoma (PDA) remains one of the least curable cancers, ranking fourth in cancer related deaths in the United States [1]. Worldwide, PDA contributes to more than 230,000 deaths annually [2]. Gemcitabine (GEM), a pyrimidine antimetabolite, is the accepted standard treatment for patients with advanced and metastatic PDA, but the benefit is small [3]. The addition of other chemotherapeutics or monoclonal antibodies or radiation to GEM has not resulted any meaningful improvement in the survival of PDA patients [4-6]. As the current therapies offer very limited benefit, there is significant unmet medical need to identify novel molecular targets and develop novel therapeutic strategies to treat this devastating disease [7].

Treatment efficacy of DNA damaging agents is determined not only by the amount of therapy-induced DNA damage but also by the capacity of tumor cells to repair the damaged DNA. Many of the conventional anticancer treatments including antimetabolites, ionizing radiation, alkylating agents, platinum compounds, and DNA topoisomerase inhibitors exert their antitumor effects by damaging the DNA in tumor cells which leads to apoptosis [8]. However, when cells are treated with DNA damaging agents, multiple checkpoints are activated, including G1, intra-S, and G2/M, leading to cell cycle arrest, thus providing time for the cell to repair thedamage and to evade apoptosis before resuming the cell cycle [9-11]. Tumor cells can exploit these repair mechanisms in response to DNA damaging chemotherapeutics, rendering tumors refractory to current therapeutic interventions. Therefore, abrogation of checkpoints function may drive tumor cells toward apoptosis and enhance the efficacy of oncotherapy.

p53 is a key regulator of the G1 checkpoint and is one of the most frequently mutated genes in cancer. Since G1 checkpoint is frequently compromised due to loss-of-function mutation of p53 gene in 50% to 70% of all cancers, the G2/M checkpoint plays a pivotal role in preventing the programmed cell death in p53-deficient tumors [12, 13]. Hence, inhibitors of the G2 checkpoint can selectively sensitize cancer cells with deficient p53 to killing by DNA-damaging anticancer agents while sparing normal tissue from toxicity.

The G2 DNA damage checkpoint ensures maintenance of cell viability by delaying progression into mitosis when cells have suffered DNA damage. Wee1 is a cellular protein kinase which inhibits Cdc2 activity, thereby preventing cells from proceeding through mitosis by maintaining G2 arrest [14]. Wee1 reversibly arrests the cell cycle by inactivating Cdc2 through phosphorylation at Tyr-15 [15]. Disruption of this phosphorylation site abrogates checkpoint- mediated regulation of Cdc2 [16]. Wee1 knockdown with siRNA has been reported to abrogate the G2 DNA damage checkpoint arrest and to sensitize cancer cells to DNA damaging agents [17]. Wee1 kinase inhibition is expected to potentiate the antitumor effect of DNA damaging chemotherapeutics by overcoming the G2 arrest and thereby promoting checkpoint bypass to facilitate the preferential killing of p53-deficient tumor cells via mitotic catastrophe.

MK-1775 is the first reported Wee1 inhibitor with high potency, selectivity, and oral bioavailability in preclinical animal models and is currently being evaluated in several phase I clinical trials [18-21]. Given that p53 mutations are common in pancreatic cancer [22], we sought to investigate efficacy and pharmacodynamic

effects of MK-1775 alone and in combination with GEM in pancreatic cancer xenografts with p53 wild-type or p53-deficient status. Our results provide compelling evidence that MK-1775 synergizes with GEM to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts. These results may help to frame clinical investigation of Wee1 inhibitors along with chemotherapy to benefit cancer patients whose tumor cells are devoid of functional p53 gene.

Materials and methods

Animals and establishment of xenografts model

Animal experiments were conducted following approval and accordance with Animal Care and Use Committee guidelines of Johns Hopkins University. Fresh pancreatic cancer specimens resected from patients at the time of surgery, with informed written patient consent, were implanted subcutaneously into the flanks of 6-week-old female nu/nu athymic mice (Harlan). The patients had not undergone chemotherapy or radiation therapy before surgery. Grafted tumors were subsequently transplanted from mouse to mouse and maintained as a live PancXenoBank according to an Institutional Review Board (IRB) approved protocol [23]. Tumor-specific mutations of protein-coding genes (exomic sequencing) in these xenografts have been recently reported [24]. Most importantly, these xenografts were not placed in culture and appear to retain most of the genetic features of the original tumor, despite serial passing across several generations of mice [25, 26].

Drugs

The Wee1 inhibitor, MK-1775, was provided by Merck Research Laboratories. GEM (Eli Lilly) was purchased from pharmacy.

In vivo efficacy experiments

Nine pancreatic cancer xenografts (6 with p53-deficient and 3 with p53 wild-type status) from PancXenoBank were allowed to grow separately on both flanks of athymic mice. When tumors reached a volume of approximately 200 mm3, mice were individually identified and randomly assigned to treatment groups, with 5 to 6 mice (8–10 evaluable tumors) in each group: (1) control; (2) MK-1775 (30 mg/kg. p.o., once daily for 4 weeks; 3) GEM (100 mg/kg, i.p., twice weekly on days 1 and 4) for 4 weeks; (4) GEM followed 24 hour later by MK-1775 in the above mentioned dose. Tumor growth was evaluated twice per week by measurement of 2 perpendicular diameters of tumors with a digital caliper. Individual tumor volumes were calculated as V ¼ ab2/2, where a being the largest diameter, b the smallest. Relative tumor growth index (TGI) on day 28 was calculated using the formula: (mean tumor volume of drug-treated group/mean tumor volume of control group) x100. Number of tumors that regressed more than 50% of its initial size in each xenograft was noted. Animals were sacrificed 1 hour after the last dose of GEM or MK-1775 and tumors were harvested for analysis except 3 mice each from GEM and combination treatment group, which were kept longer to check tumor regrowth after the treatment. Mice kept for the regrowth study were sacrificed when the tumors reached the size of control tumors in that xenograft.

Protein extraction and Western blotting

Protein extracts were prepared from tumors according to previously published method [27]. Briefly, tumors (50 mg) were minced on ice in prechilled lysis buffer containing protease cocktail (Roche Molecular Biochemicals). The minced tissue was homogenized and centrifuged at 16,000 x g at 4ºC for 10 minutes. Protein content in the supernatants was measured with the Pierce Protein Assay kit using bovine serum albumin (BSA) as a standard. Protein lysates (30 mg) were boiled in Laemmli sample buffer (Bio-Rad Laboratories) resolved by electrophoresis on 4% to 12% Bis-Tris precast gels (Bio-Rad Laboratories), and transferred to Immobilon-P membranes (Millipore). Membranes were blocked at room temperature with 5% nonfat milk (Pierce) for 1 hour. Primary antibodies against Wee1, phospho-Wee1Ser642, Cdc2 (p-Cdc2), phospho-Cdc2Tyr15 (p-Cdc2Tyr15), phospho-histone H3Ser10 (p-HH3Ser10), cleaved poly (ADP-ribose) polymeraseAsp214 (C-PARPAsp214), phospho-H2AXSer139 (g-H2AX), cellular inhibitor of apoptosis protein-2 (cIAP2) and cyclin B1 (Cell Signaling Technology) were diluted (1:1,000) in 5% BSA and incubated overnight at 4ºC with mild rocking. Membranes were probed with secondary anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (GE Healthcare) at a final dilution of 1:2,000 for 2 hours. After washing 3 times with TBST (Tris-buffered saline and Tween 20), bound antibodies were detected by enhanced chemiluminescence (GE Healthcare) or Supersignal West Femto (Pierce). Equal loading was verified with b-actin antibody.

Immunohistochemistry

Sections were prepared from formalin-fixed, paraffinembedded tumor samples. To ensure uniform handling of samples, all sections were processed simultaneously. Sections were deparaffinized by routine techniques and were quenched with 3% H2O2 for 10 minutes. The slides were steamed in citrate buffer (pH 6.0) for 20 minutes at 90ºC and were blocked with 10% FBS solution (Invitrogen) for 30 minutes prior to incubation with primary antibodies. For the p-Cdc2 staining, p-Cdc2Tyr15 antibody (Cell Signaling Technology) was used at a dilution of 1:200 followed by 1 hour incubation. For p-HH3 detection, p-HH3Ser10 antibody (Cell Signaling Technology) was used at a 1:250 dilution and incubated overnight at 4ºC. Sections were incubated with EnVision/HRP rabbit antibody for 30 minutes and diaminobenzidine (DABþ) chromogen for 10 minutes. The sections were counterstained with hematoxylin, dehydrated, and mounted. Slides were evaluated by a pathologist under 20X objective and digitized using a color camera mounted to the microscope. p-Cdc2Tyr15 and p-HH3Ser10 stained tumor cells were counted over total number of tumor cells and two sided Fisher's exact test (GraphPad Prism 5.03 software) was used for significance calculation.

Statistical analysis

All error bars are represented as the standard error of the mean. Significance was analyzed using unpaired Student's t-test. The differences were considered significant when P value was less than 0.05.

Results and discussion

The principal aim of this study was to investigate the efficacy of MK-1775 as a single agent and in combination with GEM in PDA xenografts and to assess whether the status of the p53 gene had any role in dictating treatment efficacy. We used a xenograft model which is freshly generated from the tumors taken from pancreatic cancer patients and selected nine xenografts (6 xenografts with p53-deficient and 3 xenografts with p53 wild-type status) for this study. As shown in Figure 1A, tumors in vehicletreated animals grew rapidly. Single agent MK-1775 treatment produced greater than 50% inhibition of tumor growth in 2 xenografts (PANC286 and PANC198). However, 5 of 9 xenografts treated with GEM and 6 of 9 xenografts treated with GEM plus MK-1775 produced complete tumor growth inhibition and in fact resulted tumor shrinkage compared to control and MK-1775 treated animals (Fig. 1A). These data suggest that single agent MK-1775 is unlikely to be effective in patients with PDA but that the combination of this agent with GEM has a substantial level of activity, and should be prioritized for clinical development.

Overall, none of the xenografts with p53-deficient status in the GEM treatment group produced 50% regression of initial tumor volume (Fig. 1A). However, combination of GEM and MK-1775 resulted in greater that 50% regression of initial tumor volume in 4 of 6 xenografts (66.66%) with p53-deficient status (Fig. 1A). The number of tumors that regressed more than 50% of its initial tumor size in each xenograft upon completion of treatment is provided in Table 1. Tumors with wild-type p53 status did not regress with treatments (Table 1). Among the xenografts with p53-deficient status, GEM alone treatment-induced regression in 7 of 55 tumors (12.72%), while MK-1775 in combination with GEM induced regressions in 25 of 49 tumors (51.10%) in the 6 xenografts. Tumor growth regressions in GEM plus MK-1775 treated mice were found to be significant in PANC198 ($P < 0.0001$), PANC215 ($P < 0.005$), and PANC185 ($P < 0.005$) as compared to GEM-treated mice. There was an overall 4.01-fold increase in total number of tumors regressed in the combination treatment group compared to GEM alone treatment (Fig. 1B). Overall, none of the xenografts with p53-deficient status in the GEM treatment group produced 50% regression of initial tumor volume (Fig. 1A). However, combination of GEM and MK-1775 resulted in greater than 50% regression of initial tumor volume in 4 of 6 xenografts (66.66%) with p53-deficient status (Fig. 1A). The number of tumors that regressed more than 50% of its initial tumor size in each K-ras and SMAD4 status do not influence the tumor regression pattern in the xenografts (Table 1). One limitation of preclinical studies is that the threshold of activity that translates into positive clinical outcome is not known. Often, drugs are selected for clinical development based on tumor growth inhibition in preclinical models. As our experience with freshly generated PDA models increases and more comparison data is available, we are observing that indeed only agents that result in marked tumor regressions in this model have the potential to impact patient outcome. This is illustrated by our recent work on AZD0530 and nab-paclitaxel. AZD0530, a Src kinase inhibitor, induced only modest inhibition of tumor growth in PDA xenografts and, as expected, failed in a phase II clinical trial [27, 28]. In contrast, nab-paclitaxel, in combination with GEM, resulted in marked tumor

regression in this model, which successfully predicted a positive phase II study [29, 30].

Xenograft	Genetic background			GEM	GEM + MK-1775
	P53	K-ras	SMAD4		
PANC286	WT	MUT	WT	0(8)	0(6)
PANC420	WT	WT	WT	0(10)	0(8)
JH033	WT	MUT	WT	0(8)	0(9)
PANC198	MUT	MUT	MUT	1(8)	8(8)
PANC215	MUT	MUT	WT	1(9)	5(7)
PANC185	MUT	MUT	MUT	1(10)	3(8)
PANC281	MUT	MUT	MUT	3(10)	8(10)
PANC354	MUT	WT	MUT	1(10)	0(8)
PANC374	MUT	MUT	WT	0(8)	1(8)
Total with p53 MUT				7/55 (12.72%)	25/49 (51.10%)
Total with p53 WT				0/26 (0%)	0/23 (0%)

Table 1 Mutational status and number of tumors regressed more than 50% of initial size as on day 28
NOTE: Animals were treated with vehicle, MK-1775, GEM, or GEM + MK-1775 for 4 weeks. Tumors that regressed greater than 50% of its size on day 28 in each xenograft were counted. Numbers in parenthesis denotes total number of tumors in that xenograft. Over all,7 of 55 (12.72%) tumors with p53-deficient (MUT) status regressed in the GEM treatment group. However, 25 of 49 (51.10%) of tumors with p53 MUT status regressed in the GEM + MK-1775 group. None of the tumors with p53 wild-type (WT) regressed in GEM + MK-1775 (0 of 23) or GEM (0 of 26) treatment groups. K-ras and SMAD4 status do not influence the tumor regression pattern in these xenografts.

Cdc2 initiates mitosis, which is the ultimate target of DNA replication and repair checkpoints. Chk1, Chk2, Wee1, and Myt1 are key regulators of G2 checkpoint, which act directly or indirectly to inhibit Cdc2 activity (17, 31). Chk1 and Chk2 are downstream effectors of ataxia telangiectasia-mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR), which induce G2/M cell cycle arrest by inactivating Cdc25 tyrosine phosphatases through phosphorylation [32]. Both Chk1 and Chk2 are known to phosphorylate Cdc25 on Ser216 and this phosphorylation makes Cdc25 functionally inactive [33]. Cdc25 is required for removal of inhibitory phosphotyrosines on Cdc2/cyclin B1 kinase complexes that mediate entry into mitosis. On the other hand, the inhibitory phosphorylations at Thr-14 and Tyr-15 sites of Cdc2 are mediated by Myt1 and Wee1 kinases [34, 35]. Wee1 is the major kinase phosphorylating the Tyr-15 site and Wee1-dependent phosphorylation of Cdc2 maintains the Cdc2/cyclin B1 complex in an inert form. While Myt1 preferentially phosphorylates the Thr-14 site, it can also phosphorylate the Tyr-15 site. Thus either Cdc25 inactivation and/or Wee1/Myt1 activation could contribute to G2 cell cycle arrest in response to DNA damage.

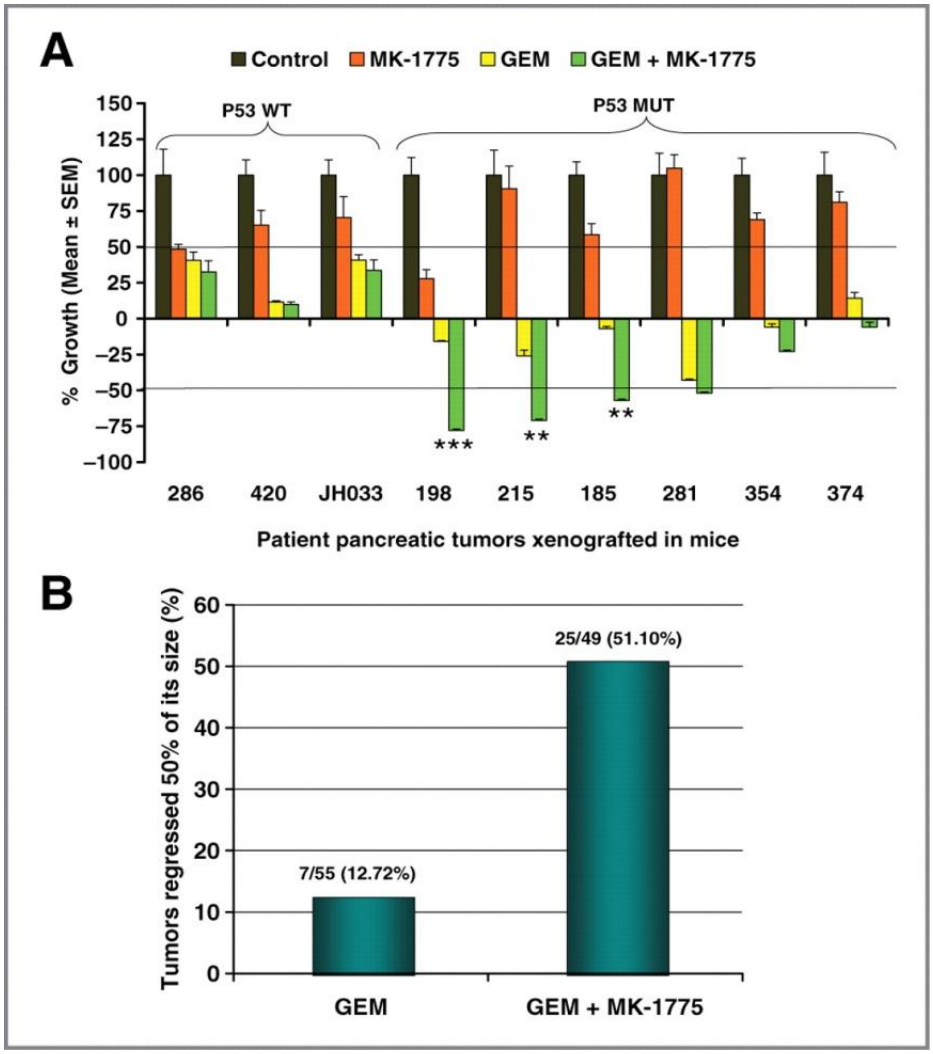


Figure 1 Combination of MK-1775 and gemcitabine potentiates the efficacy of gemcitabine in established human pancreatic cancer xenografts. Nine individual patient-derived low passage pancreatic cancer xenografts (3 with wild-type p53 (WT) and 6 with deficient p53 (MUT) were implanted in athymic mice. Animals with established tumors were dosed with MK-1775, GEM, or a combination of GEM with MK-1775 as mentioned in Materials and Methods. Tumor size was evaluated twice per week by caliper measurements. Relative tumor growth index (TGI) was calculated by relative tumor growth of treated mice (T) divided by relative tumor growth of control mice (C) _ 100. A, efficacy of MK-1775, GEM, and MK-1775, and GEM combination on the growth inhibition of pancreatic cancer xenografts. MK-1775 treatment produced greater than 50% inhibition of tumor growth in two xenografts (286, 198) compared to control tumors. Five of 9 xenografts treated with GEM and 6 of 9 xenografts treated with combination of GEM and MK-1775 produced complete tumor growth inhibition resulting in tumor shrinkage. Combination treatment caused greater than 50% regression in tumor size in 4 of 6 xenografts with p53-deficient tumors. Error bars represent SE. ***, $P < 0.0001$; **, $P < 0.005$, compared with GEM-treated tumors. B, combination therapy leads to tumor regression in p53-deficient tumors. Tumors that regressed greater than 50% of its size upon treatment on day 28 were calculated. Error bars, SE.

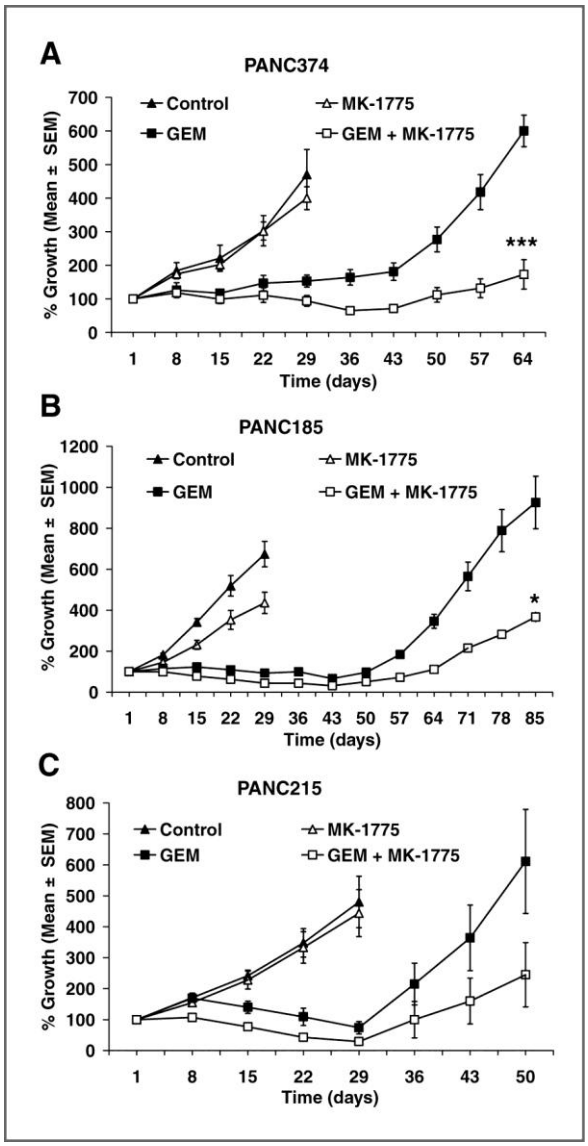


Figure 2 MK-1775 synergize with gemcitabine to inhibit growth of human pancreatic cancer xenografts. Animals were dosed with MK-1775, GEM, or a combination of GEM with MK-1775 for 4 weeks as mentioned in Materials and Methods. Tumor growth curves of (A) PANC374, (B) PANC185, and (C) PANC215 suggest that the combination of MK-1775 and GEM lead to synergistic growth inhibition. Tumors in the vehicle and MK-1775 treated animals grew progressively and were sacrificed on day 28 due to tumor burden. Animals in the GEM and combination of GEM with MK-1775 were kept longer after the 4 week treatment. Combination of GEM with MK-1775 slowed the tumor growth progression compared to GEM alone treated animals. Points, mean (n= 8 to 10 tumors per group); bars, SE. Error bars, SE. ***, $p< 0.0001$; *, $p< 0.01$, compared with GEM-treated tumors.

Chk1/2 inhibitors are in clinical development [36-38]. A recent report indicated that Chk1 is required to maintain genome integrity and cell viability, and that p53 wild-type cells are no less sensitive than p53-deficient cells to Chk1 inhibition in the presence of DNA damage. Thus, combining Chk1 inhibition with DNA damaging agents does not lead to preferential killing of p53-deficient over p53 wildtype cells, and inhibiting Chk1 does not appear to be a promising approach for potentiation of cancer chemotherapy [39]. Here we showed that Wee1 inhibition by MK-1775 could potentiate GEM sensitivity and tumor regressions, selectively in p53-deficient pancreatic cancer xenografts.

We were also interested in long-term tumor growth control and followed 3 xenografts after treatment for an extended period of time. Tumor regrowth data suggest that not only does the combination ofGEM with MK-1775 lead to synergistic tumor growth inhibition, but the effect of the combination therapy is also longer-lasting than that seen with GEM alone (Fig. 2A, B, and C). It was noteworthy, however, that tumors eventually recur, albeit at a slower pace.

In order to determine the target modulation by MK-1775, we examined Wee1, Cdc2, and their phosphorylated forms in posttreatment tumor specimens. MK-1775 treatment strongly inhibited phosphorylation of Tyr-15 of Cdc2, the primary substrate of Wee1 (Figs. 3 and 4A and C), suggesting increased Cdc2 kinase activity. In addition, the Wee1 protein was consistently reduced by MK-1775 treatment as shown by Western blotting (Fig. 3), likely due to degradation of Wee1 as MK-1775 treatment activates Cdc2 which in turn phosphorylates Wee1, ultimately leading to its ubiquitin-proteasome–dependent destruction [40].

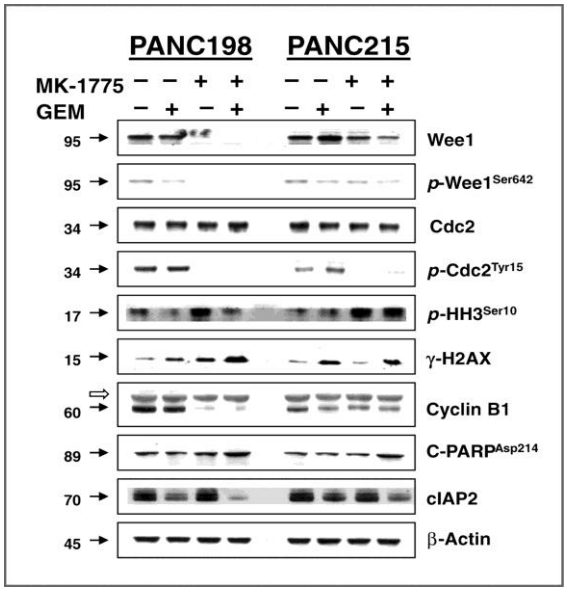


Figure 3 Combination of MK-1775 and gemcitabine inhibits Wee1 and attenuates Cdc2 phosphorylation to promote mitotic entry and apoptosis. Tumor lysates collected from vehicle, GEM, MK-1775, and combination of GEM with MK-1775 treated micewere resolved in SDS-PAGE and probed with specific antibodies against indicated proteins. MK-1775 as well as combination of MK-1775 with GEM treatment strongly inhibit Wee1 and p-Cdc2. Combination of MK-1775 and GEM treatment upregulate the expression of p-HH3, γ-H2AX, and cleaved PARP. Upon combination with GEM, MK-1775 as well as combination of MK-1775 and GEM treatment strongly inhibit the cyclin B1 expression as compared to control and GEM treatment of PANC198. Filled arrowheads indicate the molecular weight of corresponding proteins (KD). The empty arrowhead indicates the presence of a nonspecific band in cyclin B1. B-Actin was used as a loading control.

To determine whether combination therapy promotes mitotic entry, we measured the expression of p-HH3 by Western blotting as well as by immunohistochemistry. When administered in combination with GEM, MK-1775 promoted mitotic entry as measured by enhanced p-HH3Ser10 expression (Fig. 3, 4B and D). In addition, the combined treatment resulted in the upregulation of CPARP as well as downregulation of cIAP2, suggesting that combination therapy facilitates apoptotic death of tumor cells (Fig. 3). GEM, as a chain terminator, requires an active cell cycle to be effective for inhibiting tumor growth, and might induce cell cycle halt and enforce cell cycle checkpoints, which may play an important role in escalating the resistance to therapy. Thus, there is a strong rationale in combining checkpoint inhibitors with GEM as a means to enhance tumor response [41, 42]. Here we showed that GEM induces G2 arrest, which correlates with an increased Cdc2 inhibitory phosphorylation at Tyr-15 and prevents mitotic entry as evidenced by decreased p-HH3Ser10 (Fig. 4B and D). However, the decreased Cdc2 inhibitory phosphorylation at Tyr-15 caused by MK-1775 treatment indicates that MK-1775 has the ability to abrogate the G2 arrest induced by GEM and promote mitotic entry as demonstrated by enhanced p-H3Ser10 (Fig. 4B and D). Cyclin B1 was examined as a marker of G2 phase [43].

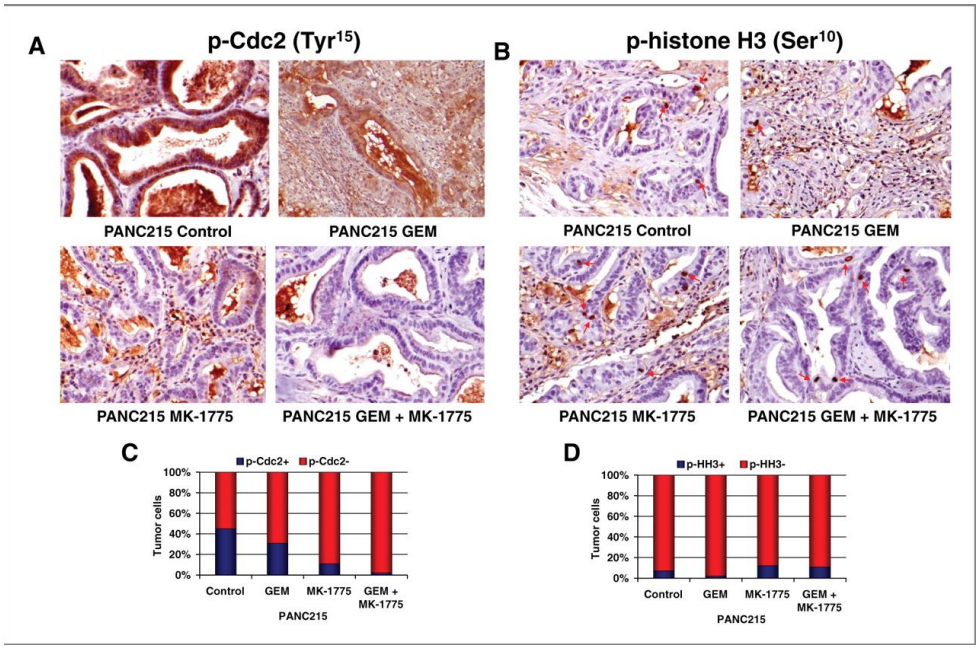


Figure 4 Immunohistochemical staining of p-Cdc2 and p=HH3. A. representative micrographs of p=Cdc2 from PANC215 showing high immunoreactivity for p-Cdc2 in the vehicle and GEM-treated tumors. MK-1775 and conbination treatment greatly inhibited the p=Cdc2 compared to vehicle and GEM-treated tumors (left). There was a 45% and 31% of tumor cells were stained positive for p=Cdc2 in the control and GEM-treated tumors, respectively, while only 11% ($P = 0.0061$ compared to GEM-treated tumors) and 2% ($P < 0.0001$ compared to GEM-treated tumors) of tumor cells were stained positive for p=Cdc2 in the MK-1775 and combination of MK-1775 and GEM-treated tumors, respectively. B, representative micrographs of p-HH3, a marker of mitosis, from PANC215 were shown in the right panel. Number of p-HH3 stained tumor cells were elevated in the MK-1775 and combination of MK-1775 and GEM-treated tumors. A total of 7% and 2% of tumor cells were stained positive for p-HH3 in the control and GEM-treated tumors, respectively, while 12% ($P = 0.0115$ compared to GEM-treated tumors) and 11% ($P = 0.0201$ compared to GEM-treated tumors) of tumor cells were stained positive for p-HH3 in the MK-1775 and combination of MK-1775 and GEM-treated tumors, respectively. C, histogram showing the number of positively stained p=Cdc2 tumors cells over total number of tumor cells. D, histogram showing the number of positively stained p=HH3 tumor cells over total number of tumor cells.

Expression of Cdc2 was not altered by treatments, while the expression of cyclin B1 was strongly inhibited by MK-1775 as well as combination of MK-1775 and GEM treatment compared to control and GEM-treated tumors of PANC198 (Fig. 3). Loss of cyclin B1 accumulation in the MK-1775 as well as combination of MK-1775 and GEM-treated tumors indicate the exit from G2 phase arrest (Fig. 3). The levels of g-H2AX were used as a surrogate for unrepaired DNA damage [44]. g-H2AX expression was clearly elevated in the combination of MK-1775 and GEM treatment group compared to GEM-treated tumors of PANC198, indicating the persistence of unrepaired DNA damage in the tumors (Fig. 3). Overall, in addition to providing mechanistic support to the observations made above, the data

provides important clues for potential biomarkers for clinical development of this drug combination.

In conclusion, our results provide compelling evidence that MK-1775 treatment leads to the inhibition and subsequent loss of Wee1 and activation of its substrate, Cdc2. The MK-1775 and GEM combination promoted the mitotic entry of tumor cells and eventually led to apoptotic death, and delayed the tumor progression compared to the GEM treatment. These findings have important clinical implications and raise the hope for potential therapeutic benefit to many PDA patients whose cancer cells are deficient for p53 function.

Reference list

1. Jemal A, Siegel R, Ward E, et al., *Cancer statistics*, 2009. CA Cancer J Clin 2009;59:225–49.
2. Li D, Abbruzzese JL. *New strategies in pancreatic cancer: emerging epidemiologic and therapeutic concepts.* Clin Cancer Res 2010;16:4313–8.
3. Burris HA, Moore MJ, Andersen J, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* J Clin Oncol 1997;15:2403–13.
4. Moore MJ, Goldstein D, Hamm J, et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* J Clin Oncol 2007;25:1960–6.
5. Van Cutsem E, Vervenne WL, Bennouna J, et al., *Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer.* J Clin Oncol 2009;27:2231–7.
6. McGinn CJ, Zalupski MM, Shureiqi I, et al., *Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer*. J Clin Oncol 2001;19:4202–8.
7. Hidalgo M. *Pancreatic cancer.* N Engl J Med 2010;362:1605–17.
8. Kawabe T. *G2 checkpoint abrogators as anticancer drugs.* Mol Cancer Ther 2004;3:513–9.
9. Elledge SJ. *Cell cycle checkpoints: preventing an identity crisis.* Science 1996;274:1664–72.
10. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, et al., *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem 2004;73:39–85.
11. Zhou BB, Elledge SJ. *The DNA damage response: putting checkpoints in perspective.* Nature 2000;408:433–9.
12. Goi K, Takagi M, Iwata S, et al., *DNA damage-associated dysregulation of the cell cycle and apoptosis control in cells with germ-line p53 mutation.* Cancer Res 1997;57:1895–902.
13. Levine AJ. *p53, the cellular gatekeeper for growth and division.* Cell 1997;88:323–31.
14. Jin P, Gu Y, Morgan DO. *Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells.* J Cell Biol 1996;134:963–70.
15. McGowan CH, Russell P. *Cell cycle regulation of human WEE1.* Embo J 1995;14:2166–75.
16. Hashimoto O, Shinkawa M, Torimura T, et al., *Cell cycle regulation by the Wee1 inhibitor PD0166285, pyrido [2,3-d] pyimidine, in the B16 mouse melanoma cell line.* BMC Cancer 2006;6:292.
17. Wang Y, Decker SJ, Sebolt-Leopold J. *Knockdown of Chk1, Wee1 and Myt1 by RNA interference abrogates G2 checkpoint and induces apoptosis.* Cancer Biol Ther 2004;3:305–13.
18. Hirai H, Arai T, Okada M, et al., *MK-1775, a small molecule Wee1 inhibitor, enhances anti-tumor efficacy of various DNA-damaging agents, including 5-fluorouracil.* Cancer Biol Ther 2010;9:514–22.
19. Hirai H, Iwasawa Y, Okada M, et al., *Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents.* Mol Cancer Ther 2009;8:2992–3000.

20. Leijen S, Beijnen JH, Schellens JH. *Abrogation of the G2 checkpoint by inhibition of Wee-1 kinase results in sensitization of p53-deficient tumor cells to DNA-damaging agents.* Curr Clin Pharmacol 2010;5:186–91.
21. Mizuarai S, Yamanaka K, Itadani H, et al., *Discovery of gene expression-based pharmacodynamic biomarker for a p53 context-specific anti-tumor drug Wee1 inhibitor.* Mol Cancer 2009;8:34.
22. Redston MS, Caldas C, Seymour AB, et al., *p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions.* Cancer Res 1994;54:3025–33.
23. Rubio-Viqueira B, Jimeno A, Cusatis G, et al., *An in vivo platform for translational drug development in pancreatic cancer.* Clin Cancer Res 2006;12:4652–61.
24. Jones S, Zhang X, Parsons DW, et al., *Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science 2008;321:1801–6.
25. Bankert RB, Egilmez NK, Hess SD. *Human-SCID mouse chimeric models for the evaluation of anti-cancer therapies.* Trends Immunol 2001;22:386–93.
26. Rubio-Viqueira B, Hidalgo M. *Direct in vivo xenograft tumor model for predicting chemotherapeutic drug response in cancer patients.* Clin Pharmacol Ther 2009;85:217–21.
27. Rajeshkumar NV, Tan AC, De Oliveira E, et al., *Antitumor effects and biomarkers of activity of AZD0530, a Src inhibitor, in pancreatic cancer.* Clin Cancer Res 2009;15:4138–46.
28. Nallapareddy S, Arcaroli J, Touban B, et al., *A phase II trial of saracatinib (AZD0530), an oral Src inhibitor, in previously treated metastatic pancreatic cancer.* J Clin Oncol 2010;28 Suppl:abstr14515.
29. Maitra A, Rajeshkumar NV, Rudek M, et al., *Nab_-paclitaxel targets tumor stroma and results, combined with gemcitabine, in high efficacy against pancreatic cancer models.* Proceedings of the 21st AACRNCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics 2009; abstr C246.
30. Von Hoff DD, Ramanathan M, Board M, et al., *SPARC correlation with response to gemcitabine (G) plus nab-paclitaxel (nab-P) in patients with advanced metastatic pancreatic cancer: a phase I/II study*. J Clin Oncol 2009;27 Suppl:abstr 4525.
31. Chaturvedi P, Eng WK, Zhu Y, et al., *Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway.* Oncogene 1999;18:4047–54.
32. Bartek J, Lukas J. *Chk1 and Chk2 kinases in checkpoint control and cancer.* Cancer Cell 2003;3:421–9.
33. Sanchez Y, Wong C, Thoma RS, et al., *Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25.* Science 1997;277:1497–501.
34. Booher RN, Holman PS, Fattaey A. *Human Myt1 is a cell cycleregulated kinase that inhibits Cdc2 but not Cdk2 activity.* J Biol Chem 1997;272:22300–6.
35. Parker LL, Sylvestre PJ, Byrnes MJ III, et al., *Identification of a 95-kDa WEE1-like tyrosine kinase in HeLa cells.* Proc Natl Acad Sci U S A 1995;92:9638–42.
36. Morgan MA, Parsels LA, Zhao L, et al., *Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint and inhibition of homologous recombinational DNA repair.* Cancer Res 2010;70:4972–81.
37. Dai Y, Grant S. *New insights into checkpoint kinase 1 in the DNA damage response signaling network.* Clin Cancer Res 2010;16:376–83.
38. Perona R, Moncho-Amor V, Machado-Pinilla R, et al., *Role of CHK2 in cancer development.* Clin Transl Oncol 2008;10:538–42.

39. Zenvirt S, Kravchenko-Balasha N, Levitzki A. *Status of p53 in human cancer cells does not predict efficacy of CHK1 kinase inhibitors combined with chemotherapeutic agents*. Oncogene 2010;29:6149–59.
40. Watanabe N, Arai H, Nishihara Y, et al., *M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP.* Proc Natl Acad Sci U S A 2004;101:4419–24.
41. Zhou BB, Bartek J. *Targeting the checkpoint kinases: chemosensitization versus chemoprotection.* Nat Rev Cancer 2004;4:216–25.
42. Matthews DJ, Yakes FM, Chen J, et al., *Pharmacological abrogation of S-phase checkpoint enhances the anti-tumor activity of gemcitabine in vivo.* Cell Cycle 2007;6:104–10.
43. Scott IS, Morris LS, Rushbrook SM, et al., *Immunohistochemical estimation of cell cycle entry and phase distribution in astrocytomas: applications in diagnostic neuropathology.* Neuropathol Appl Neurobiol 2005;31:455–66.
44. Morgan MA, Meirovitz A, Davis MA, et al., *Radiotherapy combined with gemcitabine and oxaliplatin in pancreatic cancer cells.* Transl Oncol 2008;1:36–43.

Chapter 10

Pancreatic ductal adenocarcinoma in hereditary diffuse gastric cancer. A case report

Niki A. Ottenhof, Roeland F. de Wilde, Folkert H. M. Morsink, Wendy W. J. de Leng, Margreet Ausems, Hans Morreau, Richard van Hillegersberg, G. Johan A. Offerhaus, Anya N. Milne

Human Pathology 2012; 43: 457-461

Abstract
Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant cancer syndrome characterized by highly penetrant diffuse gastric cancer. It is caused by germline mutations in *CDH1,* encoding the cell-cell adhesion protein E-cadherin. Pancreatic ductal adenocarcinoma (PDAC) is one of the most dismal malignancies in humans. Although absent E-cadherin expression in PDAC is related to a higher tumor grade and a worse prognosis, there have been no reports of PDAC associated with HDGC. Here we describe a patient with HDGC who was subsequently diagnosed with PDAC. To investigate if the previously identified *CDH1* germline mutation initiated PDAC development we performed mutational and proteomic analysis. We conclude that the PDAC did not occur in the context of the germline *CDH1* mutation but rather appeared as a sporadic event. Immunohistochemistry ultimately proved to be the most valuable tool of investigation as persistent *CDH1* staining in the PDAC unequivocally revealed E-cadherin expression.

Introduction

Hereditary diffuse gastric cancer

Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant cancer syndrome [1]. It is characterized by highly penetrant diffuse gastric cancer (DGC). The risk of developing DGC for patients suffering from HDGC is estimated to be 67% in men and 83% in woman by the age of 80 [2]. The *CDH1* gene is the only gene known to be associated with HDGC and in approximately 25-50% of HDGC patients a germline mutation in *CDH1* is identified. Approximately 100 different germline mutations in *CDH1* have been described in over 150 HDGC families [3, 4].

Decreased or lost E-cadherin expression is found in several different malignancies and this has been linked to multiple unfavorable features in these malignancies, among which increased invasion/migration and poor prognosis [5]. Patients with HDGC are advised to undergo a prophylactic total gastrectomy because of the high penetrance, the aggressive growth rate of DGC and the difficulty to detect even advanced stage tumors with surveillance endoscopy [6]. This operation usually takes place within the third decade of life.

Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is one of the most dismal malignancies in humans with a 5-year survival of less than 5% and a median survival of only 6 months [7]. Because most patients present with locally advanced or distant metastatic disease, treatment with curative intent is often not a possibility. Surgical resection remains the only curative treatment but only 10-20% of PDAC patients qualify for this. A number of high incidence mutations have been described in PDAC. The most commonly occurring mutations are found in the *KRAS2* oncogene and the tumor suppressor genes *CDKN2A /p16*, *TP53* and *SMAD4* [8]. Loss of E-cadherin expression in PDAC is related to a higher tumor grade and a worse prognosis [9]. However, there have been no reports where PDAC was found to be associated with HDGC. Moreover, there is as yet no evidence for an increased risk of PDAC in patients with a germline *CDH1* mutation.

Here, we describe a patient with the HDGC syndrome with a previously identified *CDH1* germline mutation and multifocal intramucosal diffuse gastric carcinoma in the prophylactic gastrectomy specimen, who was subsequently diagnosed with PDAC. The question arose as to whether the pancreatic tumor was related to the germline mutation in *CDH1*. In order to answer this question we performed immunohistochemistry in both the gastric tumors as the pancreatic tumor. Also, extensive mutation and methylation analysis was performed in the search for the ‘second genetic hit’ that possibly eliminated *CDH1* signaling.

Materials and methods

For analysis paraffin blocks of both the gastric tumors and the PDAC were obtained. Neoplastic cells from the PDAC were isolated via manual microdissection. With use of laser microdissection (Carl Zeiss, Jena, Germany) the malignant cells were isolated from the lamina propria of the gastrectomy specimen.

Normal gastric epithelium and a non-malignant lymph node were included as control samples. Genomic DNA was isolated using the Qiamp DNA minikit (Qiagen, Netherlands) according to manufacturers' protocol or, in the case of the gastric cells, by direct lyses with ProteinaseK (Roche, Basel, Switzerland).

Immunohistochemistry (IHC)
Immunohistochemistry was performed on formalin fixed paraffin embedded tissue samples as previously described [10]. Two different E-cadherin antibodies were used: E-cadherin antibody, dill 1:100, (ab1416; Abcam, Cambridge, UK) which is directed at the extracellular part of the protein (exon-8) and E-cadherin, clone 36, dill 1:200, (BD Bioscience, USA) which binds the cytoplasmic domain.

E-cadherin mutation analysis
To confirm the earlier identified germline mutation and possibly identify a somatic mutation in the WT-allele, the complete *CDH1* gene was sequenced in the DGC. Sequencing was performed as previously described [10]. Forward and reverse primers for each exon were designed (sequences available upon request). The sequencing reaction mix was run on the 3130 Genetic Analyzer (Applied Biosystems, USA). Sequences were analysed using the CODONCODE ALIGNER software.

Loss of heterozygosity (LOH)
To evaluate LOH of E-cadherin one microsatellite marker was used, located on chromosome 16q22 (D16s2624) around the *CDH1* locus. PCR was performed (primer sequences and cycling conditions available upon request). Analysis was carrier out using an automated ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) using the GeneScan™ 500 ROX™ Size Standard and the manufacturer's Genescan® 3.1.2 software (Applied Biosystems, USA). LOH was assumed if the allelic imbalance factor was greater than 1.6 or less than 0.6.

Methylation specific PCR (ms-PCR)
To evaluate promotor methylation of the *CDH1* gene the Epitect Bisulfite kit was used (Qiagen, Venlo, Netherlands) according to the protocol provided by the manufacturer. After bisulfite treatment, PCR reactions were performed. Primers specific for either methylated or unmethylated templates were designed (sequences and cycling conditions available upon request). Sequencing was performed as described above.

Methylation specific multiplex-ligand dependent probe-amplification (ms-MLPA)
Because the ms-PCR did not produce reliable results another methylation assay was performed. For this method the SALSA MLPA KIT ME004-A1 tumour suppressor kit (MRC-Holland, Amsterdam, The Netherlands) was used. This kit contains 31 probes that recognize CpG Islands in 27 different tumor suppressor genes among which *CDH1*. Analysis was performed as described by the manufacturer. Genescan analysis was performed as described above and samples were analysed using the Cofalyzer software (MRC Holland).

TP53/KRAS2 mutation analysis
To evaluate mutation status of *TP53* and *KRAS2* in the PDAC sample, sequencing was performed. Primers for exon 4-9 of *TP53* and exon 1-2 of *KRAS2* were designed (sequences and PCR cycling conditions available upon request). PCR products were purified using a combination of exonuclease I (Westburg, Leusden, The Netherlands) and shrimp alkaline phosphatase (GE Healthcare, Sweden). Sequencing was performed as described above and analysed using Mutation Surveyor (Softgenetics, State College, PA, USA).

Results
Clinical findings
A 56 year old man presented at the emergency room with intense epigastrical pain, diarrhea and darkened urine. Physical examination revealed a palpable liver 2 fingers under the ribcage and jaundice, both on skin and sclera. Patient history reported a prophylactic total gastrectomy 2 years prior, related to an earlier identified germline mutation in *CDH1*. Pathological examination of the resected stomach had revealed multiple diffusely growing poorly differentiated mucus producing adenocarcinomas, varying in size from 1-8mm and limited to the lamina propria. Four family members had died from gastric cancer between the ages of 43 and 56. No pancreatic malignancies were diagnosed in the patient's family. Mutation analysis of *CDH1* performed previously identified a mutation in exon 10, 1565 +2insT.

A CT-scan was performed which revealed enlarged bile- and pancreatic ducts and a mass in the head of pancreas, suspicious for a malignant pancreatic tumor. A Whipple's pancreatoduodenectomy was performed. Examination of the resected head of pancreas revealed a 3 cm large mass which on microscopic examination turned out to be a moderate to poorly differentiated pancreatic ductal adenocarcinoma . The malignant process extended into the duodenum wall and was present <1mm from the duodenal resection margin. All other resection margins were tumor free. Three of the 16 excised lymph nodes contained malignant cells, warranting the staging T3N1Mx or IIB. Adjuvant therapy was initiated consisting of several runs of gemcitabine. However, 3 months later a CT-scan revealed a recurrent lesion in the pancreas and multiple lesions in the liver, suspicious for metastatic tumors. Therefore all treatment was ceased. The patient was treated for his pain and discharged to home where he passed away 2 months later.

When the diagnosis PDAC was first confirmed, clinicians and family members questioned whether the development of this tumor was related to the *CDH1* germline mutation. To confirm or contradict this, Immunohistochemistry (IHC) was performed. Although the results from the IHC were almost conclusive, additional mutation analysis was performed to identify the exact 'second genetic hit' in the gastric samples. Also, to further characterize the PDAC, mutation analysis was performed on the pancreatic tumor samples.

Immunohistochemistry (IHC)

To evaluate E-cadherin expression in the gastric tumors and in the PDAC, IHC was performed. Two different E-cadherin monoclonal antibodies were used. The different gastric tumors showed the same labeling pattern. Cytoplasmic staining was granular and focally positive in all samples. Membranous staining was present, though clearly diminished compared to the normal epithelium surrounding the malignant cells. Exon-8 skipping as a second genetic hit, often observed in sporadic DGC [11], became unlikely as one of the E-cadherin antibodies specifically recognized exon-8. The decrease in membranous signaling strongly suggested a defect in E-cadherin signaling in the gastric lesions (Figure 1C/D). In the PDAC sample, E-cadherin staining was strong with both antibodies and there was no decrease compared to normal epithelial cells suggesting intact E-cadherin signaling (Figure 1E/F).

Mutation analysis
All 16 exons of *CDH1* were sequenced. This did not identify any new mutations. The previously located germline mutation was confirmed in all samples. The mutation was located at the splice-site of exon 10 and intron 10 and consisted of an insertion of a T-nucleotide just 2 bases after exon 10: 1565+2insT, as shown in Figure 1A.

Loss of heterozygosity (LOH)
To further specify the defect in E-cadherin signaling, LOH analysis was performed. The allelic imbalance factor was calculated at 1,21 for the gastric tumor samples and 1,00 for the PDAC sample suggesting the absence of LOH. In addition, LOH could be ruled out because mutation analysis of *CDH1* revealed a heterozygous SNP in exon 13 (RS1801552) in both the gastric and the pancreatic samples.

Methylation analysis
The next step in search of the mechanism for somatic inactivation of the WT-allele in the gastric tumors was to ascertain the presence or lack of methylation of the promotor, the most common 'second genetic hit' in HDGC [12]. A methylation specific PCR was performed but this did not produce reliable results. Therefore, another technique was tried; ms-MLPA. Unfortunately, although this reaction was repeated multiple times it did not result in interpretable data either, presumably as the amount of DNA obtained from the gastric cells was not sufficient to perform further testing. No E-cadherin promoter methylation was found in the PDAC.

Analysis of the PDAC for KRAS2 and *TP53*
Because no decrease in E-cadherin staining was seen with IHC in the PDAC samples, we hypothesized that this was a sporadic malignancy, unrelated to the germline defect in *CDH1*. Because the genetic alterations that take place in PDAC development are well documented we tried to confirm this hypothesis by performing mutation analysis of the frequently mutated genes, *KRAS2* and *TP53*. *KRAS2* mutation analysis revealed a point mutation in exon 1, codon 12; G12D. This mutation is often observed in pancreatic cancers and results in a constitutively active protein. In *TP53* a mutation was found as well. Sequencing of exon 7 revealed the TAT-TGT missense mutation at codon 238, responsible for Cys to Tyr

substitution (C238T). These results combined with the normal E-cadherin expression seen on IHC, strongly suggests that the PDAC was a sporadic tumor which developed due to somatic mutations in *KRAS2* and *TP53*.

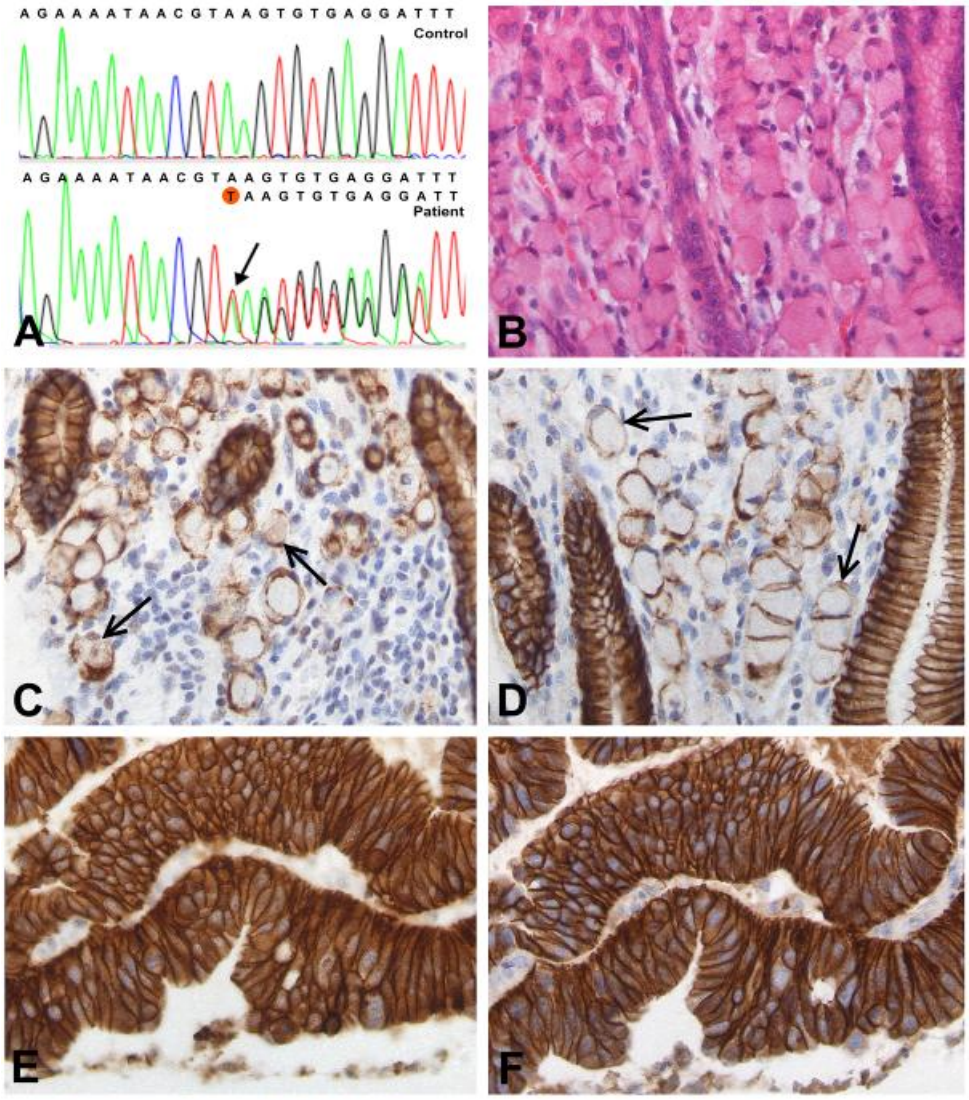


Figure 1 A: Mutation analysis *CDH-1* showing a mutation in exon 10, 1565 +2insT in the patient. Arrow indicates mutation. B: Hematoxilin stained gastric resection specimen clearly depicting the SRCC,C en D: E-cadherin IHC on SRCC, using two different antibodies both showing a clear decrease in membranous staining intensity in the signet ring cells compared to the normal gastric epithelium; granular cytoplasmic staining is present in the tumor cells. E and F: E-cadherin IHC on the PDAC using two different antibodies showing strong membranous as well as cytoplasmic staining suggesting unaltered E-cadherin expression in the malignant cells.

Discussion

Decreased E-cadherin expression has been described in multiple different types of tumors, among which pancreatic cancer. However, apart from colorectal cancer and lobular breast cancer no data has been published claiming that patients with HDGC have a higher chance of developing malignant tumors. This suggests that sole loss of E-cadherin expression is not sufficient to initiate tumorigenesis in the pancreas. Nevertheless, there is a strong suggestion that diminished E-cadherin expression increases invasive potential and worsens prognosis [9]. Therefore it seemed plausible that the germline mutation in *CDH1* played a role in the development of the pancreatic tumor in the patient presented in this manuscript.
Because IHC showed diminished membranous E-cadherin expression in the gastric tumor samples, we tried to identify the mechanism for somatic knock out of the WT-allele. Unfortunately, this was not possible. Sequencing showed only the previously known germline mutation. LOH was also not the cause for the decrease in E-cadherin expression. Methylation analysis was incomplete due to technical difficulties. Therefore, methylation of the promotor could not be ruled out as a 'second hit' although the persistent yet decreased E-Cadherin staining in the gastric tumors on immunohistochemistry suggest that post translational modifications are more likely to have caused somatic knock out.

The *CDH1* pattern of staining observed with IHC has been described previously. In 2009, Humar and colleagues found residual activity of E-cadherin in HDGC patients, even with a 'second genetic hit' consisting of promoter methylation [13]. Barber and colleagues described their search for the mechanism of somatic inactivation of the wild-type allele and found persistent but reduced staining in almost all cases. Remarkably, one of their cases exhibited a mutation at almost the exact same location as the patient described above, 1565+1G>T and like the patient described above, extracellular E-cadherin labelling was decreased but no LOH or promotor methylation was identified [14].

Given the evidence from our experiments, we ruled out the possibility that aberrant E-cadherin signalling influenced the development of the PDAC. This conclusion can be drawn solely upon the results from the IHC, and is strongly supported by the molecular analyses carried out. The alterations found in *KRAS2* and *TP53* are characteristic for sporadic PDACs. The persistent E-cadherin staining in IHC with no evidence of a decrease in intensity clearly showed the presence of the wild-type-allele in the PDAC sample, thereby excluding *CDH1* as a PDAC tumor inducer.

As shown by other groups, identifying the 'second genetic hit' remains difficult in HDGC and this study emphasizes once more the value of IHC. Although more elaborate and refined techniques have been developed, we highlight the vital importance and relevance of IHC which, with use of a reliable antibody, enables a conclusion to be drawn on protein expression as well as on protein location. When multiple antibodies are used, one can even distinguish between alterations in the extracellular and cytoplasmic domain of the protein. Although the exact mechanism for E-cadherin loss was not identified, the knowledge that this did not influence the

PDAC development is crucial for both family and scientist. It seems advisable to continue the search for other mechanisms of wild-type-allele knock out in HDGC. Also, IHC should be further explored to possibly develop antibodies that differentiate better between the different genetic alterations because, still nowadays, IHC holds the answer to otherwise unanswered questions.

Reference list

1. Guilford P, Hopkins J, Harraway J, et al., *E-cadherin germline mutations in familial gastric cancer.* Nature, 1998. **392**(6674): p. 402-5.
2. Pharoah PD, Guilford P and Caldas C, *Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families.* Gastroenterology, 2001. **121**(6): p. 1348-53.
3. Guilford P, Humar B and Blair V, *Hereditary diffuse gastric cancer: translation of CDH1 germline mutations into clinical practice.* Gastric Cancer, 2010. **13**(1): p. 1-10.
4. Ghaffari SR,Rafati M, Sabokbar T, et al., *A novel truncating mutation in the E-cadherin gene in the first Iranian family with hereditary diffuse gastric cancer.* Eur J Surg Oncol, 2010.
5. Takeichi M, *Cadherins in cancer: implications for invasion and metastasis.* Curr Opin Cell Biol, 1993. **5**(5): p. 806-11.
6. Lewis FR, Mellinger JD, Hayashi A, et al., *Prophylactic total gastrectomy for familial gastric cancer.* Surgery, 2001. **130**(4): p. 612-7; discussion 617-9.
7. *Cancer Facts and Figures*. 2009, American Cancer Society: Atlanta, GA.
8. Ottenhof NA, Milne AN, Morsink FH, et al., *Pancreatic intraepithelial neoplasia and pancreatic tumorigenesis: of mice and men.* Arch Pathol Lab Med, 2009. **133**(3): p. 375-81.
9. Joo YE, Rew JS, Park CS, et al., *Expression of E-cadherin, alpha- and beta-catenins in patients with pancreatic adenocarcinoma.* Pancreatology, 2002. **2**(2): p. 129-37.
10. Sitarz R, Leguit RJ, de Leng WW, et al., *Cyclooxygenase-2 mediated regulation of E-cadherin occurs in conventional but not early-onset gastric cancer cell lines.* Cell Oncol, 2009. **31**(6): p. 475-85.
11. Berx G, Becker KF, Hofler H, et al., *Mutations of the human E-cadherin (CDH1) gene.* Hum Mutat, 1998. **12**(4): p. 226-37.
12. Machado JC, Oliveira C, Carvalho R, et al., *E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma.* Oncogene, 2001. **20**(12): p. 1525-8.
13. Humar B, Blair V, Charlton A, et al., *E-cadherin deficiency initiates gastric signet-ring cell carcinoma in mice and man.* Cancer Res, 2009. **69**(5): p. 2050-6.
14. Barber M, Murrell A, Ito Y, et al., *Mechanisms and sequelae of E-cadherin silencing in hereditary diffuse gastric cancer.* J Pathol, 2008. **216**(3): p. 295-306.

Chapter 11

Analysis of LKB1 mutations and other molecular alterations in pancreatic acinar cell carcinoma

Roeland F. de Wilde, Niki A. Ottenhof, Marnix Jansen, Folkert H. M. Morsink, Wendy W. J. de Leng, G. Johan A. Offerhaus, Lodewijk A. A. Brosens

Modern Pathology 2011; 24: 1229-1236

Abstract

Acinar cell carcinoma is a rare non-ductal neoplasm of the pancreas with poorly defined molecular genetic features. Recently, biallelic inactivation of LKB1 was described in an acinar cell carcinoma of a Peutz-Jeghers patient carrying a heterozygous germline LKB1 mutation, and inhibition of mTOR signaling resulted in partial remission of the tumor. To explore the potential of mTOR inhibitors in sporadic acinar cell carcinoma, the LKB1 gene was investigated in five sporadic acinar cell carcinomas by sequence analysis, methylation analysis and mRNA expression. In addition, microsatellite instability and methylation of a number of tumor suppressor genes were investigated and KRAS, TP53, CDKN1A, SMAD4 and CTNNB1 were studied by mutation analysis and immunohistochemistry. No mutations, deletions or promoter hypermethylation of LKB1 were found in any of the sporadic acinar cell carcinomas, and mRNA expression of LKB1 was not altered. Amplifications at chromosome 20q and 19p were found in 100 and 80% of the cases, respectively. In addition, hypermethylation of one or more tumor suppressor genes was found in 80% of cases. One case harbored a TP53 mutation, and expression of SMAD4 and CTNNB1 was altered in one case each. No KRAS mutations or microsatellite instability were found. To conclude, no evidence for a role for LKB1 in tumorigenesis of sporadic pancreatic acinar cell carcinoma was found. However, copy number variations and hypermethylation were found in a majority of cases. Molecular pathways involved in acinar cell carcinoma-tumorigenesis differ from those involved in ductal pancreatic neoplasms. Further studies are needed to increase our understanding of molecular pathogenesis of acinar cell carcinoma, which may eventually result in development of new therapeutic targets.

Introduction

Acinar cell carcinoma is a rare, non-ductal neoplasm of the exocrine pancreas derived from acinar cells. It represents about 1–2% of all exocrine pancreatic neoplasms [1]. The mean age at diagnosis is 62 years, although several pediatric cases have been described [2–5]. The vast majority (85–90%) of pancreatic exocrine neoplasms are pancreatic ductal adenocarcinomas, for which several morphologically distinct precursor lesions have been identified, including pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasm, and mucinous cystic neoplasm [6]. With a mean survival of 18 months and a 5-year survival rate of less than 10%, the prognosis of acinar cell carcinoma is slightly better than that of pancreatic ductal adenocarcinoma [2,3]. Histologically, acinar cell carcinomas are often well circumscribed and sometimes multinodular lesions composed of large nodules of cells separated by hypocellular fibrous bands. A desmoplastic stroma reaction, characteristic for pancreatic ductal adenocarcinoma, is usually lacking. Neoplastic cells are characterized by a minimal to moderate amount of granular cytoplasm and round to oval uniform nuclei [2,3,5]. Identification of pancreatic enzymes by immunohistochemistry, such as trypsin, chymotrypsin and lipase can be helpful in establishing the diagnosis [2,3]. In some patients, hypersecretion of lipase by the neoplasm can produce a distinctive syndrome of subcutaneous fat necrosis and polyarthralgia. Molecular features of pancreatic acinar cell carcinoma have not been well characterized, but, in contrast to pancreatic ductal adenocarcinoma, acinar cell carcinomas rarely show KRAS, TP53, or SMAD4 mutations [2,7–12]. APC or CTNNB1 mutations have been found in a subset (23.5%) of acinar cell carcinomas [10]. Recently, acinar cell carcinoma of the pancreas was described for the first time in a patient with Peutz-Jeghers syndrome and biallelic inactivation of LKB1 allele was demonstrated in this tumor, indicating a causative role for LKB1 in the development of this neoplasm. Moreover, treatment with an mammalian target of rapamycin (mTOR) inhibitor resulted in partial remission of the tumor [13]. Peutz-Jeghers syndrome is a rare autosomal dominant disorder caused by germline mutation of LKB1 and characterized by mucocutaneous melanin pigmentation and distinctive non-neoplastic hamartomatous intestinal polyps [14]. Peutz-Jeghers syndrome patients are at increased risk for gastrointestinal and non-gastrointestinal malignancies, including pancreatic cancer. The relative risk of pancreatic cancer in Peutz-Jeghers syndrome has been estimated to be as high as 132 and the lifetime risk 36% [15]. Reported cases of pancreatic cancer in Peutz-Jeghers syndrome mainly involve pancreatic ductal adenocarcinoma and mucinous cystadenocarcinoma, but serous cystadenoma, intraductal papillary mucinous neoplasms, and papillary adenoma of the pancreatic duct have also been reported [16–19]. Moreover, LKB1 has previously been shown to have a role in the pathogenesis of intraductal papillary mucinous neoplasms, sporadic pancreatic, and biliary carcinomas [17,20]. LKB1 is involved in the establishment of cell polarity and in the regulation of cellular response to energy and stress. Specifically, LKB1 activates adenosine monophosphate-activated protein kinase (AMPK) among other kinases, and leads to inhibition of mTOR. mTOR is a highly conserved serine/threonine kinase that regulates protein synthesis and cell growth. Loss of LKB1 function, therefore, leads

to increased mTOR signaling and increased cell growth and is a treatment-specific target of interest [14]. In view of the recent report of an acinar cell carcinoma with second hit inactivation of LKB1 in a Peutz-Jeghers syndrome patient, our aim was to further investigate molecular alterations in acinar cell carcinomas and, specifically, to investigate whether LKB1 is involved in acinar cell carcinoma carcinogenesis.

Methods

Patient samples and DNA isolation

Archival formalin-fixed paraffin-embedded tissue was obtained from the Peutz-Jeghers syndrome patient (PJS1) described by Klümpen et al [13], and five confirmed sporadic pancreatic acinar cell carcinoma cases (T1–5) from the surgical pathology archives of the Academic Medical Center Amsterdam (n¼3), Onze Lieve Vrouwe Gasthuis Amsterdam (n¼1) and University Medical Center Utrecht (n¼1). A panel of immunohistochemical markers, including vimentin, keratin markers, trypsin, chymotrypsin, lipase, CD10, CD56, synaptofysin, chromogranin and NSE, was used in establishing the diagnosis (data not shown). The study was carried out according to the guidelines and with approval of the ethics committee of these institutions. Formalin-fixed paraffin-embedded tissue sections were used for immunohistochemistry analysis and genomic DNA isolation. DNA was isolated after deparaffinization using the QIAamp DNA mini Kit (Qiagen). DNA concentrations were determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). Frozen tumor tissue was available from three cases (T2–4) and corresponding normal pancreatic tissue of cases T2 and T3 (T2_NP1/NP2 and T3_NP1). In addition, five independent normal frozen pancreatic tissue specimens (C1–5) were available from the tissue bank of the University Medical Center Utrecht.

Mutation analysis

LKB1 mutation analysis was carried out in all tumor samples using primers for all coding exons and exon/intron boundaries of the LKB1 gene, as previously described [21]. Sequence analysis of exons 1 and 2 of KRAS and exons 4–9 of TP53 was carried out in all tumor samples. Primer sequences and PCR conditions are available on request. After purification, the sequence reaction was carried out using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Samples were run on a 3730 genetic analyzer (Applied Biosystems) and analyzed using CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA).

(Methylation specific) multiplex ligation-dependent probe amplification ((ms-MLPA)

Copy number variations in the LKB1 gene were studied using the MLPA kit P101 (MRC-Holland, Amsterdam, The Netherlands) as previously described [21]. Four genomic DNA samples derived from peripheral blood from healthy individuals served as normal controls, to which control probes and samples were normalized. Deletions and duplications were defined as ratios of o0.5 and 41.75, respectively, and were repeated at least two times [21].

CpG island promoter methylation status of LKB1 and 24 other tumor suppressor (including mTOR pathway genes PTEN, TSC2, and VHL) was investigated using the ms-MLPA ME002-B1 tumor suppressor kit (MRC-Holland), according to manufacturer's protocol. Two negative control samples containing unmethylated DNA and one positive control containing completely methylated DNA were analyzed simultaneously. Samples were run on an ABI 3730 genetic analyzer and results were analyzed using the MRC Coffalyser software (MRC-Holland). Methylation was defined as a percentage higher than 25%. All samples were analyzed in duplicate.

LKB1 mRNA expression

Quantitative analysis of LKB1 mRNA expression levels was assessed in three of the acinar cell carcinomas, of which frozen tissue was available (cases T2–4). Corresponding normal pancreatic tissue of two acinar cell carcinoma cases (T3_NP1/NP2 and T4_NP1) and five normal pancreata (C1–5) were included to serve as normal controls. The housekeeping gene GAPDH was used as an internal control for reverse transcription in all samples. mRNAwas isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNAwas prepared from 1 mg mRNA using Superscript III (Invitrogen) and a combination of random hexamers and oligo-(dT) primers (Invitrogen).

RT-PCR for LKB1 and GAPDH was performed using an Assay on Demand (LKB1: Hs00176092_m1, GAPDH: Hs99999905_m1, Applied Biosystems) according to the manufacturers protocol in the ABI7900 (Applied Biosystems). Samples were tested in duplicate. Relative expression was determined using SDS2.2.1 software (Applied Biosystems).

Microsatellite analysis

DNA was isolated by direct lysis using ProtK after microdissection of tumor and normal formalin-fixed paraffin-embedded tissue of the same patient. Next, tumor and normal DNA were PCR-amplified using six markers recommended by the National Cancer Institute to screen for microsatellite instability in hereditary non-polyposis colorectal cancer (so called Bethesda markers, BAT25, BAT26, BAT40, D17S250, D2S123, and D5S346) [22]. After PCR amplification, 1 ml of each product was added to 0.5 ml of GS500 size marker and 23.5 ml MilliQ. The amplified fragments were separated by denaturing gel electrophoresis using an ABI 3730 genetic analyzer (Applied Biosystems). Data were analyzed using GeneMapper software (Applied Biosystems). Microsatellite instability was defined as the presence of extra or shifted bands in comparison with the normal control. The tissues were classified as microsatellite high instability, if instability was noted in 30% of the loci investigated, microsatellite low instability, if instability was observed in o30% of the loci investigated, andmicrosatellite stable, if there was no instability. Primer sequences and PCR conditions are available on request.

Immunohistochemistry

Immunohistochemistry on all formalin-fixed paraffin-embedded tissue samples for CTNNB1, TP53, and SMAD4 was performed and scored as described before [23]. CDKN1A immunohistochemistry was performed using a monoclonal antibody

(Invitrogen, 18-0401; 1:150) according to the TP53/SMAD4 protocol and results were scored with respect to intensity (negative, weak, or positive). Immunohistochemistry of mismatch repair proteins MSH6, MSH2,MLH1, PMS2 was performed in the Leica Bond III fully automated immunohistochemistry stainer. Detailed conditions are available on request. All slides were scored by an experienced GI pathologist (GJAO).

Results

LKB1 Sequence Analysis and MLPA

All nine coding exons and exon/intron boundaries of the LKB1 gene were analyzed by sequence analysis and MLPA in all five acinar cell carcinoma cases (T1–5). Sequence analysis did not reveal any alterations in LKB1 and MLPA analysis did not show deletions in the LKB1 gene. Amplification of LKB1 was found in four cases, including three samples (T1, T2 and T5) with complete amplification of chromosome 19 containing LKB1. GATA5 amplification was found in all five cases. BRCA2 and RB1 were amplified in one case (Table 1).

		Copy number analysis: amplification				**Methylation**				
Case	**Age/sex**									
T1	M/48	*GATA5 (20q13.3)*	*LKB1* (19p13.3)*			*ESR1*	*MSH6*		*THBS1*	*CDH13*
T2	V/68	*GATA5 (20q13.3)*	*LKB1* (19p13.3)*			*ESR1*			*GSTP1*	
T3	M/61	*GATA5 (20q13.3)*								
T4	V/19	*GATA5 (20q13.3)*	*LKB1 (19p13.3)*	*BRCA2 (13q13.1)*	*RB1§ (13q14.2)*	*ESR1*		*PAX5*	*PAX6*	*WT1*
T5	M/33	*GATA5 (20q13.3)*	*LKB1* (19p13.3)*				*MSH6*	*PAX5*	*CD44*	
Total		**5/5**	**4/5**			**3/5**	**2/5**	**2/5**		

Table 1 Summary of copy number and methylation analysis by (methylation specific)-MLPA. * chromosome 19 amplification; § - amplification of two probes.

Methylation-specific MLPA

CpG island promoter methylation of LKB1 and 24 other tumor suppressor genes was studied using ms-MLPA in all five acinar cell carcinoma cases (T1–5) (Table 1). No hypermethylation of the LKB1 promoter was found in any of the samples. However, three tumors showed hypermethylation of the ESR1 promoter and two showed hypermethylation of MSH6 and PAX5. CD44, CDH13, GSTP1, PAX6, THBS1, and WT1 were hypermethylated in one tumor.

LKB1 quantitative mRNA analysis

Quantitative mRNA analysis did not show significant differences in LKB1 mRNA expression between corresponding normal and tumor and normal control samples (Figure 1), which is in line with the results of the mutation and MLPA analysis. Frozen tissue was available from one patient, with chromosome 19 amplification

(T2) and one with LKB1 amplification (T4) but did not show aberrant LKB1 mRNA expression.

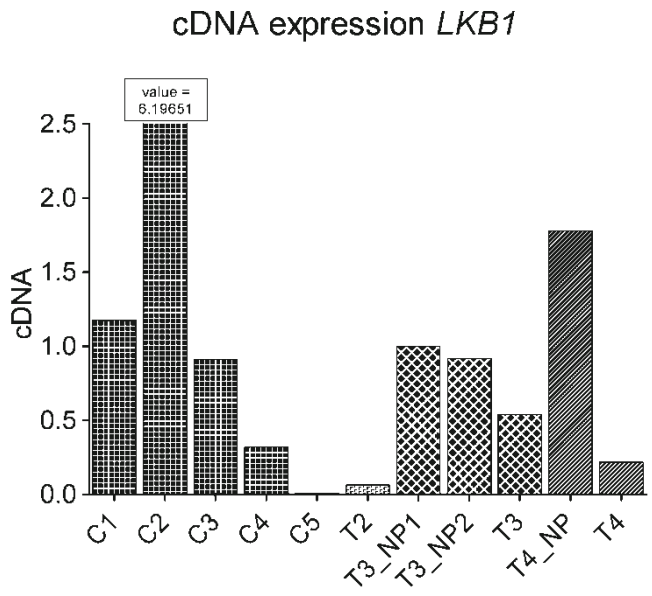


Figure 1 Relative LKB1 cDNA expression levels in five normal control pancreata (c1-5) and three pancreatic acrinar cell carcinomas (T2-4). NP: normal pancreas.

KRAS and TP53 mutation analysis

Mutation analysis of KRAS did not show any mutations. TP53 mutation analysis revealed two mutations in exon 6 in case T1 (one silent mutation in codon 213 (R213R) and one missense mutation in codon 217 (V217M)) (Figure 2c). KRAS and TP53 mutation analysis could not be completed in case T5 because of insufficient DNA (Table 2).

Immunohistochemistry of TP53, CTNNB1, SMAD4 and CDKN1A

Results of immunohistochemistry are summarized in Table 2. TP53 immunolabeling showed nuclear accumulation in case T1, which is in accordance with the mutation that was found in this case (Figures 2a and c). Aberrant expression of CTNNB1 and SMAD4 was found in two different cases (case T2 and T3, respectively) (Figures 3a and b). Immunolabeling for CDKN1A showed variable staining intensity from positive to absent (Figures 2b and 3c). Interestingly, positive CDKN1A staining was found in the case harboring the TP53 mutation (Figure 2b).

Microsatellite instability and immunohistochemistry of mismatch repair genes

All tumors were microsatellite stable and showed normal expression of the mismatch repair proteins MLH1, MSH2, MSH6, and PMS2. However, one case (PJS1) showed loss of heterozygosity (LOH) of D17S250 and D5S346 and one case (T3) showed LOH of D17S250 (Table 2).

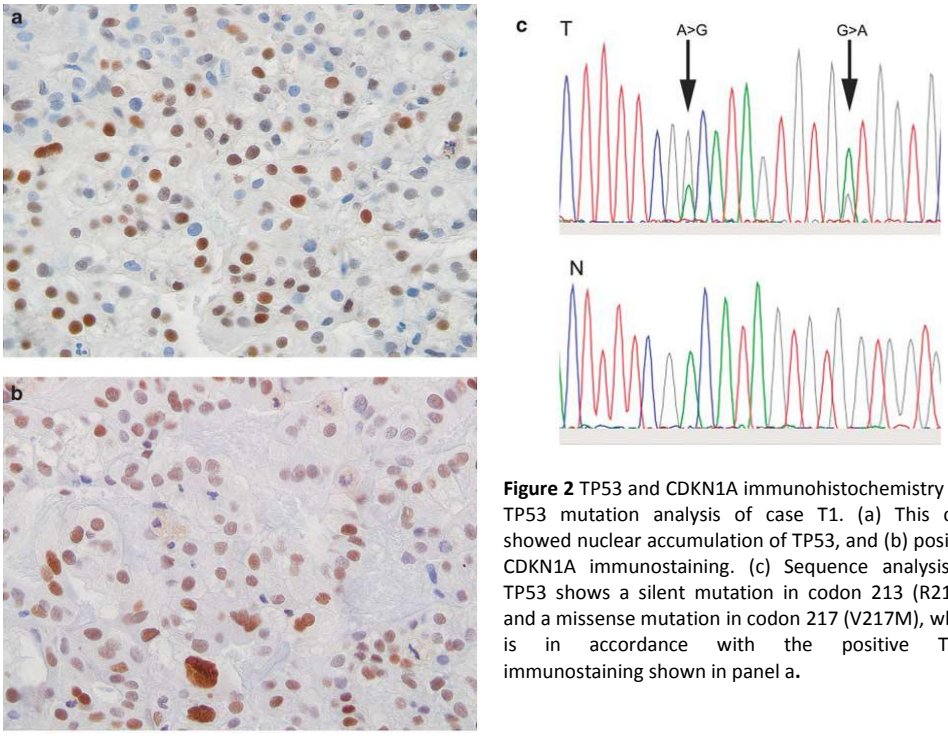


Figure 2 TP53 and CDKN1A immunohistochemistry and TP53 mutation analysis of case T1. (a) This case showed nuclear accumulation of TP53, and (b) positive CDKN1A immunostaining. (c) Sequence analysis of TP53 shows a silent mutation in codon 213 (R213R) and a missense mutation in codon 217 (V217M), which is in accordance with the positive TP53 immunostaining shown in panel a.

		Mutation analysis		**Immunohistochemistry**				**MSI**	**LOH**
Case	**Sex, age**	*p53*	K-*ras*	p53	p21	SMAD4	ß-Catenin		
PJS1	M, 45	WT	WT	-	0	+	N	MSS	D17S250, D5S346
T1	M, 48	S & MS	WT	+	2	+	N	MSS	
T2	V, 68	WT	WT	-	0	+	F	MSS	
T3	M, 61	WT	WT	-	2	-	N	MSS	D17S250
T4	V, 19	WT	WT	-	0	+	N	MSS	
T5	M, 33	NA*	NA*	-	1	+	N	MSS	

Table 2 Summary of *K-ras* and *p53* mutation analysis, microsatellite analysis and immunohistochemistry. For p53: -,normal negative labeling; +, positive labeling. For p21: 0, absent labeling; 1, weak positive labeling; 2, strong positive labeling. For SMAD4: -, absent labeling; +, normal positive labeling. For ß-Catenin: N, normal membraneous (negative); F, focal nuclear labeling (positive). * insufficiënt DNA. Abbreviations: PJS, Peutz-Jeghers syndrome; WT, wild type; S, silent mutation; MS, missense mutation; MSI, microsatellite instability; MSS, microsatellite stable; LOH, loss of heterozygosity; NA, not applicable.

Discussion

Acinar cell carcinoma is a rare non-ductal neoplasm of the exocrine pancreas and associated with a poor prognosis. Molecular alterations in acinar cell carcinoma carcinogenesis are poorly characterized. Only few studies investigated molecular alterations in a limited number of acinar cell carcinomas (Table 3). These studies

do not reveal specific molecular alterations that characterize acinar cell carcinoma carcinogenesis, although it does become clear that genes commonly involved in pancreatic ductal adenocarcinoma such as KRAS, TP53, and SMAD4 do not have key roles in acinar cell carcinoma carcinogenesis. In addition, one study reported Wnt pathway activation in 24% of acinar cell carcinoma cases and copy number variations have been reported by two investigators [2,7–12,24]. CpG island promoter methylation has not been studied in acinar cell carcinoma before.

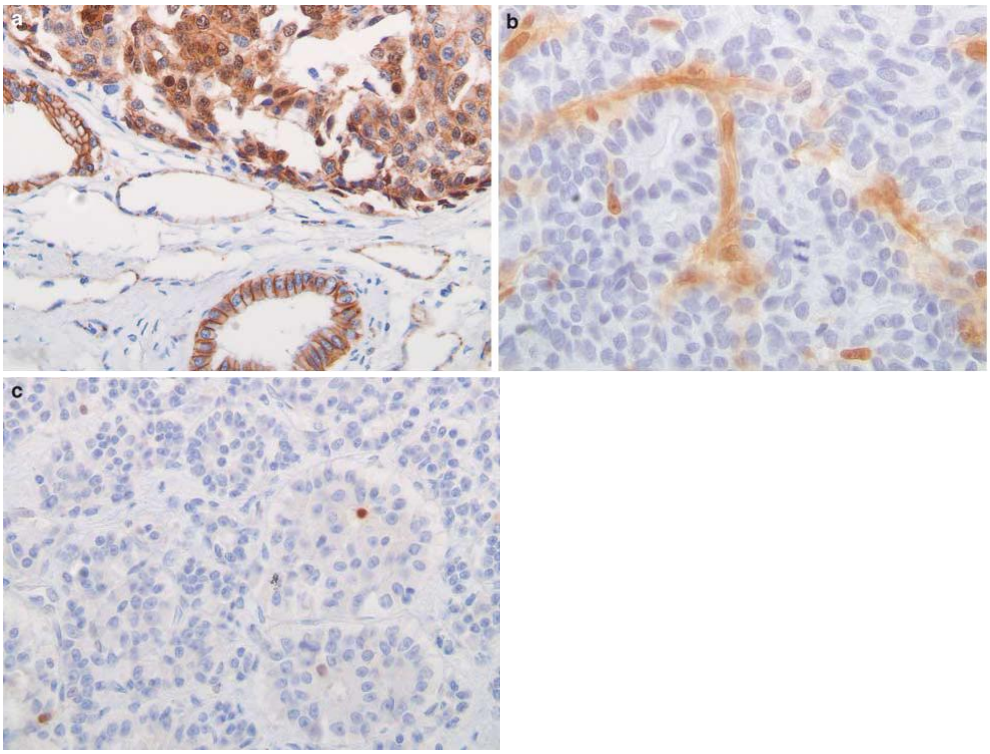


Figure 3 Microphotographs of immunohistochemistry. (a) Nuclear expression of CTNNB1 in an acinar cell carcinoma (T2). (b) Loss of SMAD4 expression in an acinar cell carcinoma (T3). (c) Negative CDKN1A expression in an acinar cell carcinoma (T4).

Recently, acinar cell carcinoma of the pancreas was described for the first time in a patient with Peutz-Jeghers syndrome [13]. A causal relationship of Peutz-Jeghers syndrome, caused by heterozygous germline mutation of the LKB1 gene, and this acinar cell carcinoma was demonstrated by showing loss of the wild-type LKB1 allele in the tumor. In addition, a partial response of the tumor on treatment with an mTOR inhibitor was shown, which fits with the notion of complete LKB1 inactivation leading to aberrant mTOR signaling in this tumor [14]. The mTOR inhibitor sirolimus was previously also reported to suppress and prevent polyp growth in murine Peutz-Jeghers syndrome models and tumor growth in a mouse model for acinar cell carcinoma.25–28 Moreover, alterations in mTOR signaling are found in

a subset (14%) of pancreatic neuroendocrine tumors and LKB1 inactivation is found in pancreatic neoplasms of ductal origin [17,20,29].

Genetic alteration	**Cases studied**	**Abnormal cases**	**% Abnormal**	**References**
K-ras	36	1	3%	2, 7, 8, 11
P53	69	0	0%	2, 7, 9-12
P16/CDKN2A mutation	6	0	0%	11
SMAD4	35	0	0%	10-12
APC/ ß-catenin	17	4*	24%	10
MSI	13	3#	23%	10
Gain at 1q, 12p, Xq,	6		50-60%	32
Loss at 16p, 16q	6		50%	32
Loss at 1p, 4q, 17p	9		>70%	12
Loss at 11q, 13q, 15q, 16q	9		60-70%	12
Loss at 3q, 6q, 8q, 18q, 21q			50-60%	12
11p loss	12		50%	10

Table 3 Summary of molecular alterations in pancreatic acinar cell carcinomas reported in the literature. * 1 *ß-catenin* mutation, 3 *APC* mutations. # 1 MSI-high, 2 MSI-low.

On the basis of these observations, our aim was to investigate whether LKB1 inactivation is involved in tumorigenesis of sporadic pancreatic acinar cell carcinomas, and to address the potential role for mTOR inhibitors in treatment of sporadic acinar cell carcinoma. However, we did not find inactivation of LKB1 by somatic mutation, deletion, or promoter hypermethylation in five sporadic acinar cell carcinomas. This was further substantiated by the finding of normal LKB1 mRNA expression levels in three studied cases. Moreover, no methylation or deletions were found in three other mTOR pathway genes (PTEN, TSC2, and VHL) by (ms-)MLPA. Although investigated in a limited number of cases, these results do not point to a role for LKB1 or disturbed mTOR signaling in the tumorigenesis of sporadic acinar cell carcinomas.

Interestingly, studies using conditional knockout mice have shown that targeted deletion of Lkb1 and k-ras in the pancreas leads to development of ductal neoplasms. Hezel et al. [30] showed that loss of Lkb1 in murine pancreas results in disturbed acinar cell polarity, abnormal cytoskeletal organization, loss of tight junctions, and inactivation of AMPK/MARK/SAD kinases, eventually resulting in postnatal acinar cell degeneration, acinar-to-ductal metaplasia, and pancreatic serous cystadenomas. Moreover, Habbe et al. [31] showed that selective mutation of k-ras in acinar cells results in murine pancreatic intraepithelial neoplasia of all histological grades.

In addition, alterations in genes commonly involved in pancreatic ductal adenocarcinoma carcinogenesis, including TP53, KRAS, and SMAD4 were investigated. Our results confirm previous observations that these genes do not have a major role in acinar cell carcinoma. One acinar cell carcinoma showed a TP53 mutation and one case showed loss of SMAD4 protein expression, which has not been reported previously (Table 3). Interestingly, one acinar cell carcinoma showed P53-independent expression of CDKN1A, a cyclin-dependent kinase inhibitor also known as WAF1/CIP1. Although CDKN1A has not been investigated in acinar cell carcinoma previously, this phenomenon has been described in pancreatic ductal adenocarcinoma [32].

Alterations in the APC/CTNNB1 pathway have been reported in 4 of 17 (23.5%) acinar cell carcinoma cases [10]. In our series, one patient demonstrated nuclear expression of CTNNB1 (20%) indicating activated Wnt signaling. Although we did not find aberrant expression of mismatch repair proteins or microsatellite instability, others have reported microsatellite instability in a minority (23%) of acinar cell carcinomas [10].

Previous studies have shown a relatively high degree of genomic copy number variations in acinar cell carcinoma [12,24]. Allelic loss has been reported in 460% of acinar cell carcinomas of chromosome arms 1p, 4q, 11q, 13q, 15q, 16p, 16q and 17p and gain at arms 1q, 12p and Xq [12,24]. Using MLPA, we identified gains of GATA5 at 20q13.3 (5/5 cases, 100%), LKB1 at 19p13.3 (4/5 cases, 80%), and at 13q (BRCA2 and RB1 locus) in one case (20%). As these are all tumor suppressor genes, amplifications of these genes most likely reflects the genomic instability in these tumors instead of being events that drive tumorigenesis, and are therefore regarded as an epiphenomenon. In addition, LOH at D17S250 was found in two cases and LOH at D5S346 in one case.

CpG island promoter methylation has not been studied in pancreatic acinar cell carcinoma previously. We identified hypermethylation of one or more tumor suppressor genes in 4/5 (80%) of cases (Table 1). One of these genes (THBS1), methylated in one acinar cell carcinoma, has previously been described to be methylated in pancreatic ductal adenocarcinomas (7%) and intraductal papillairy mucinous neoplasms (12%) [33].

To conclude, our results do not point to a role for LKB1 in acinar cell carcinoma pathogenesis. Although other genes involved in mTOR signaling, such as VHL, PTEN, TSC2, and PIK3CA, may be mutated, currently there seems no rationale for treatment of sporadic acinar cell carcinoma with mTOR inhibitors. We did not find specific molecular alterations in acinar cell carcinoma, but copy number variations and hypermethylation are frequently encountered. Moreover, we confirm that molecular pathways involved in acinar cell carcinoma-tumorigenesis differ from those involved in ductal pancreatic neoplasms. Our results form an incentive for more elaborate evaluation of molecular alterations in acinar cell carcinoma, which

may eventually lead to development of new therapeutic targets and improved prognosis of this disease with a dismal prognosis.

Reference list

1. Morohoshi T, Held G, Klöppel G, *Exocrine pancreatic tumours and their histological classification. A study based on 167 autopsy and 97 surgical cases.* Histopathology 1983;7:645–661.
2. Hoorens A, Lemoine NR, McLellan E, et al., *Pancreatic acinar cell carcinoma. An analysis of cell lineage markers, p53 expression, and Ki-ras mutation.* Am J Pathol 1993;143:685–698.
3. Klimstra DS, Heffess CS, Oertel JE, et al., *Acinar cell carcinoma of the pancreas. A clinicopathologic study of 28 cases*. Am J Surg Pathol 1992;16: 815–837.
4. Luttges J, Stigge C, Pacena M, et al., *Rare ductal adenocarcinoma of the pancreas in patients younger than age 40 years.* Cancer 2004;100:173–182.
5. Webb JN. *Acinar cell neoplasms of the exocrine pancreas.* J Clin Pathol 1977;30:103–112.
6. Hruban RH, Maitra A, Kern SE, et al., P*recursors to pancreatic cancer.* Gastroenterol Clin North Am 2007;36:831–849.
7. Pellegata NS, Sessa F, Renault B, et al., *K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions*. Cancer Res 1994;54:1556–1560.
8. Terhune PG, Heffess CS, Longnecker DS. *Only wildtype c-Ki-ras codons 12, 13, and 61 in human pancreatic acinar cell carcinomas.* Mol Carcinogenesis 1994; 10:110–114.
9. Terhune PG, Memoli VA, Longnecker DS. *Evaluation of p53 mutation in pancreatic acinar cell carcinomas of humans and transgenic mice.* Pancreas 1998;16: 6–12.
10. Abraham SC, Wu TT, Hruban RH, et al., *Genetic and immunohistochemical analysis of pancreatic acinar cell carcinoma: frequent allelic loss on chromosome 11p and alterations in the APC/beta-catenin pathway.* Am J Pathol 2002;160:953–962.
11. Moore PS, Orlandini S, Zamboni G, et al., *Pancreatic tumours: molecular pathways implicated in ductal cancer are involved in ampullary but not in exocrine nonductal or endocrine tumorigenesis.* Br J Cancer 2001;84:253–262.
12. Rigaud G, Moore PS, Zamboni G, et al., *Allelotype of pancreatic acinar cell carcinoma.* Int J Cancer 2000;88:772–777.
13. Klumpen HJ, Queiroz KC, Spek CA, et al., *mTOR inhibitor treatment of pancreatic cancer in a patient with Peutz-Jeghers syndrome.* J Clin Oncol 2011;29:e150–e153.
14. Jansen M, Ten Klooster JP, Offerhaus GJ, et al., *LKB1 and AMPK family signaling: the intimate link between cell polarity and energy metabolism.* Physiol Rev 2009;89:777–798.
15. Giardiello FM, Brensinger JD, Tersmette AC, et al., *Very high risk of cancer in familial Peutz-Jeghers syndrome.* Gastroenterology 2000;119:1447–1453.
16. Giardiello FM, Welsh SB, Hamilton SR, et al., *Increased risk of cancer in the Peutz-Jeghers syndrome*. N Engl J Med 1987;316:1511–1514.
17. Sato N, Rosty C, Jansen M, et al., *STK11/LKB1 Peutz-Jeghers gene inactivation in intraductal papillarymucinous neoplasms of the pancreas.* Am J Pathol 2001;159:2017–2022.
18. Canto MI, Goggins M, Hruban RH, et al., *Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study.* Clin Gastroenterol Hepatol 2006;4:766–781; quiz 665.
19. Yee NS, Furth EE, Pack M. *Clinicopathologic and molecular features of pancreatic adenocarcinoma associated with Peutz-Jeghers syndrome.* Cancer Biol Ther 2003;2:38–47.

20. Su GH, Hruban RH, Bansal RK, et al., *Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers.* Am J Pathol 1999;154:1835–1840.
21. de Leng WW, Jansen M, Carvalho R, et al., *Genetic defects underlying Peutz-Jeghers syndrome (PJS) and exclusion of the polarity-associated MARK/Par1 gene family as potential PJS candidates.* Clin Genet 2007;72:568–573.
22. Boland CR, Thibodeau SN, Hamilton SR, et al., *A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer.* Cancer Res 1998;58:5248–5257.
23. Langeveld D, van Hattem WA, de Leng WW, et al., *SMAD4 immunohistochemistry reflects genetic status in juvenile polyposis syndrome.* Clin Cancer Res 2010;16:4126–4134.
24. Taruscio D, Paradisi S, Zamboni G, et al., *Pancreatic acinar carcinoma shows a distinct pattern of chromosomal imbalances by comparative genomic hybridization.* Genes Chromosomes Cancer 2000;28:294–299.
25. Shackelford DB, Vasquez DS, Corbeil J, et al., *mTOR and HIF-1alpha-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome.* Proc Natl Acad Sci USA 2009;106:11137–11142.
26. Robinson J, Lai C, Martin A, et al., *Oral rapamycin reduces tumour burden and vascularization in Lkb1 (+/-) mice.* J Pathol 2009;219:35–40.
27. Wei C, Amos CI, Zhang N, et al., *Chemopreventive efficacy of rapamycin on Peutz-Jeghers syndrome in a mouse model.* Cancer Lett 2009;277:149–154.
28. Zhang N, Lyons S, Lim E, et al., *A spontaneous acinar cell carcinoma model for monitoring progression of pancreatic lesions and response to treatment through noninvasive bioluminescence imaging.* Clin Cancer Res 2009;15:4915–4924.
29. Jiao Y, Shi C, Edil BH, et al., *DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors.* Science 2011;331:1199–1203.
30. Hezel AF, Gurumurthy S, Granot Z, et al., *Pancreatic LKB1 deletion leads to acinar polarity defects and cystic neoplasms.* Mol Cell Biol 2008;28:2414–2425.
31. Habbe N, Shi G, Meguid RA, et al., *Spontaneous induction of murine pancreatic intraepithelial neoplasia (PanIN) by acinar cell targeting of oncogenic Kras in adult mice.* Proc Natl Acad Sci USA 2008;105: 18913–18918.
32. DiGiuseppe JA, Redston MS, Yeo CJ, et al., *p53-independent expression of the cyclin-dependent kinase inhibitor p21 in pancreatic carcinoma.* Am J Pathol 1995;147:884–888.
33. Sato N, Ueki T, Fukushima N, et al., *Aberrant methylation of CpG islands in intraductal papillary mucinous neoplasms of the pancreas.* Gastroenterology 2002;123:365–372.

Chapter 12

Summary and concluding remarks

Pancreatic ductal adenocarcinoma (PDAC) is a very aggressive disease. Most patients present with locally advanced or distant metastatic disease and tumors are often irresectable which leads to the extremely short average survival time of only 5 months. Mortality almost equals incidence and every year more than 37.000 people die from PDAC in the USA. Despite extensive research performed over the past decades, these numbers have only slightly changed. Although many advances have been made in understanding the genetic alterations that govern tumor development, these insights have not yet been translated to more effective treatment strategies. Currently, gemcitabine remains the standard first line treatment after resection, but the effects of treatment are marginal.

When evaluating PDAC development, genetics play a featured role. Alterations in genes and protein expression lead to changes in cellular features involved in cell division, migration, invasion and angiogenesis. Investigations of these changes have led to the current understanding of pancreatic carcinogenesis. It is now well established that PDAC develops through a step-wise progression in which pre-invasive lesions precede malignant transformation. This suggests that there is a window of opportunity for early diagnosis and treatment. Studying the genetic alterations that drive invasive tumor progression may lead to the discovery of molecular markers as diagnostic or treatment target. Moreover, the different genes and proteins involved in prognosis of PDAC are of specific interest, not only for determining the outlook of each specific patient but also because these markers can be new targets for directed treatment.

Chapter 3 of this thesis reviews the progression from normal pancreatic epithelial cell to PDAC. In the pancreas, the malignant transformation to invasive carcinoma is preceded by the development of pre-invasive lesions, called pancreatic intraepithelial neoplasia (PanIN). Based on the morphological abnormality of these PanIN lesions they can be classified into different stages. These stages of PanIN lesions are accompanied by a specific set of genetic mutations including alterations in oncogenes, tumor-suppressor genes and genome-maintenance genes. Moreover, the development of the genetically-engineered mouse models of ductal type invasive pancreatic cancer has enhanced our understanding of the disease considerably. These genetically-engineered mouse models faithfully recapitulate the human transformation from normal pancreatic ductal cell to PDAC. In both mice and men, the *KRAS2* oncogene seems to be the initiator of malignant transformation. Inactivation of tumor suppressor genes like *CDKN2A* and/or *TP53* accelerates tumor progression. Although differences remain, using mouse models to investigate novel treatment modalities for human pancreatic cancer has great potential and can be considered as first step leading to new treatment options in the near future.

Chapter 4 discusses the genetic alterations found in malignant tumors of the pancreas focusing on most commonly affected pathways instead of most commonly affected genes. Recently, 12 signaling pathways were identified which

show alterations in most, if not all, PDACs and the aim of this chapter was to describe the cellular functioning of these pathways and the genes involved, focusing specifically on their role in pancreatic carcinogenesis. The complexity of the genetics accompanying PDAC indicates that it is impossible to design a treatment that fits all. With regard to personalized treatment, it seems advisable to use the genetic alterations as guide for the best possible treatment for a specific patient. Future research is needed for the understanding of the interplay between the different genetic pathways in tumor aggressiveness and therapy resistance.
Although mean survival of PDAC patients is extremely short, a small number of patients survive much longer than expected and why this is, is not understood. Evaluation of prognostic characteristics in PDAC is therefore relevant. In **Chapter 5,** we investigated the characteristics that affect prognosis in PDAC. Over the last decades, many clinicopathological features have been claimed to affect survival in PDAC, but multivariate analysis is usually not conducted in these studies. To evaluate these features and to investigate their individual prognostic potential, a cohort was constructed consisting of both short-term en long-term survivors of PDAC. Multivariate analysis was conducted investigating the effects on survival of both clinical and histological features. The study showed that lymph node ratio <0.2, tumor-free resection margins and positive Smad4 expression were independent variables that positively affected survival. It is concluded that when patient-specific prognosis is determined, these characteristics should be included.
A number of prognosticators of PDAC have been described, but in most cases these features do not explain long-term survival. In **Chapter 6,** a unique group of these long-term survivors of PDAC is described. For this report, a cohort was formed of very long-term survivors (VLTS) of PDAC, defined as >10-year survival after resection of the tumor, which is a rarity. To examine whether the VLTS tumors showed the same general genetic alterations as the tumors of short-term survival patients, the VLTS tumors were screened for the most commonly affected genes *KRAS2* and *TP53*. Also, earlier reported genetic prognosticators such as Smad4 expression, microsatellite instability and dCK/hENT1 expression were investigated to evaluate whether these characteristics could possibly explain prolonged survival. Surprisingly, in VLTS and short-term survivors, none of the earlier identified prognostically-relevant features showed a significant difference. Moreover, the number of tumors showing mutant *TP53* and *KRAS2* signaling did not differ from numbers in the literature, leading to the conclusion that the VLTS tumors developed in a similar manner as the short-term survivor tumors.

In search for other factors involved in prolonging survival in PDAC, the VLTS cohort described in chapter 6 was further investigated in **Chapter 7**. Five VLTS patients were selected for quantative proteomics to examine whether protein expression in the VLTS tumors differed from a short-term survival control group. Several proteins were found to be differentially expressed in the VLTS tumors, one of which was galectin-1, a protein involved in apoptosis and this protein was further investigated. When evaluating expression in a cohort of sporadic PDACs by immunohistochemistry, galectin-1 was found to be expressed in the stromal

compartment of the tumors and not in the neoplastic epithelial cells. The stromal galectin-1 was closely but inversely associated with pancreatic cancer survival with a significantly lower expression in the VLTS cohort. Further investigation of the exact role of galectin-1 in PDAC development and progression is warranted. Also, the proteomic analysis resulted in a number of other proteins differentially expressed in the VLTS group and these are currently under investigation for their possible value in PDAC diagnosis and treatment.

One of the signaling pathways often affected in PDAC is the embryonic signaling pathway Notch. Notch signaling is vital for the embryonic development of the pancreas but Notch signaling is inactive in the adult pancreas. However, Notch signaling is re-activated in many PDACs. Therefore, it is argued that inhibition of Notch signaling has potential as treatment. In **Chapter 8,** we investigated whether inhibition of one of the Notch receptors, the Notch1-receptor, using a monoclonal receptor-specific antibody, would affect survival, growth rate or anchorage-independent growth of pancreatic cancer cells. Earlier studies that used less specific inhibitors of Notch showed promising results *in vitro*. Treatment of two selected PDAC cell lines with the Notch-1 antibody induced a reduction in anchorage-independent growth. However, Notch-1 inhibition did not affect survival or growth rate. These findings are in contrast with earlier reports on Notch-1 inhibition in PDAC but comparison of our findings with those in the other reports is not possible as methods of Notch inhibition varied. Although Notch may be an important treatment target for PDAC, determination of the exact effects of inhibition of the different components of the Notch signaling pathway is critical before introducing Notch-inhibition as a new treatment in a clinical setting.

In 1997, gemcitabine was implicated as first line treatment after resection of PDAC. Since then, multiple treatment strategies have been proposed. Addition of other chemotherapeutic agents, radiation or targeted treatment approaches showed promising results *in vitro* but were disappointing in the clinical setting. Recently FOLFIRINOX, a combination of infusional 5-FU/folinic acid, irinotecan and oxaliplatin was proven to improve survival in metastatic disease in a phase-III trial. However, gemcitabine is still the first choice in locally advanced or surgically resectable disease. In **chapter 9,** MK-1775, a potent Wee1 inhibitor, was shown to synergize with gemcitabine in treating pancreatic cancer cells transplanted in athymic mice. This effect was only observed in tumors that exhibited mutations in *TP53*, a tumor suppressor gene affected in 50-75% of all PDACs. Earlier reports showed that inhibition of Wee1 results in mitotic catastrophe due to cell cycle progression induced by inhibition of the protein, thereby enlarging tumor cell death. This effect is especially pronounced in tumors without functioning p53 since these tumors rely on Wee1 to stop cell cycle progression. Moreover, MK-1775 targets the extensive stromal compartment of PDACs and has been shown to enhance drug delivery to the tumor cells. These results indicate that Wee1 inhibition could enhance the effectivity of gemcitabine treatment which may result in prolonged survival after resection of the primary tumor. Actual tumor regression, as observed

with MK-1775, has been shown to effect survival in clinical phase II studies and MK-1775 should therefore be considered for application in a clinical setting.

Hereditary cancer syndromes are characterized by the presence of a germline mutation in a cancer-associated gene. For many cancer syndromes, the genes responsible for the disease are known and in the past the study of these genes has led to new insights in the genetics involved in carcinogenesis of a specific cell type. On the other hand, the tumor spectrum associated with specific hereditary cancer syndromes, is not always well defined. In **Chapter 10,** we describe a patient that was diagnosed with a hereditary diffuse gastric carcinoma caused by a germline mutation in the *CDH1* gene, and was subsequently diagnosed with PDAC. We determined whether the germline mutation caused the development of the pancreatic carcinoma as well. After evaluation of the *CDH1* status using immunohistochemistry, it was concluded that the wild-type allele of *CDH1* was intact in the pancreatic tumor. The tumor developed as an incidental carcinoma, independently of the hereditary diffuse gastric cancer syndrome. Immunohistochemistry appeared to be an easy and inexpensive method to rule out that the PDAC in this patient was part of the tumor spectrum of the germline mutation in *CDH1*.

Although the main focus of this thesis was on PDAC, other types of pancreatic cancer exist. **Chapter 11** discusses a rare type of pancreatic cancer, acinar cell carcinoma (ACC). These tumors represent 1-2% of all exocrine pancreatic tumors. Because of their low incidence not much is known about its development. In view of a recent report of an ACC with second hit inactivation of *LKB1* in a Peutz-Jeghers syndrome patient, our aim was to further investigate molecular alterations in ACCs and, specifically, to investigate whether *LKB1* is involved in the carcinogenesis of ACC. Of the 5 ACC's evaluated, none exhibited alterations in the *LKB1* gene leading to the conclusion that *LKB1* is not generally involved in ACC development. The results of this study also confirmed previous observations that the genes typically involved in PDAC development do not have a major role in ACC development. More research is needed to understand carcinogenesis of ACCs.

The studies described in this thesis show that genetic and proteomic alterations are the main forces that drive pancreatic tumor development. Understanding these alterations is the first step on the road to improve the current outlook of patients diagnosed with pancreatic cancer. The signaling pathways affected by these alterations and their interplay show the larger picture of the effects on cellular functioning and should be the target for treatment. The Notch pathway is one of these potential targets although the exact role of this pathway in PDAC development is not clear. Inhibition of the Notch pathway in a clinical setting is at this stage premature. Targeting the stromal compartment of the tumor is another new promising strategy that may improve the grim outlook of PDAC patient. Wee1

inhibition in combination with gemcitabine treatment in a clinical trial may be a next step.

It is clear that there is still a long way to go for finding a cure for this devastating disease but we are progressing slowly. For treatment of a disease as complex and aggressive as pancreatic cancer, developing a clear understanding of how the disease originates and evolves is imperative. Over the past 20 years this has been largely established. For future years, it is hoped that these insights will result in improvement of treatment and survival and hopefully the results of the research described in this thesis contribute to that.

Chapter 13

Nederlandse samenvatting
Summary in Dutch

Het menselijk lichaam is opgebouwd uit cellen. In deze cellen bevinden zich genen, waarin de informatie ligt die nodig is voor de bouw en het functioneren van cellen in het lichaam. Cellen functioneren namelijk dankzij eiwitten en de genen bevatten de bouwtekening voor deze eiwitten. Menselijke cellen bevatten ruim 20.000 genen. Als er fouten optreden in de genen komen er constructiefouten in de eiwitten waardoor verschillende celprocessen in het geding kunnen komen. In gezonde cellen vinden vele processen plaats waar eiwitten bij betrokken zijn. Een groot aantal van deze processen zijn kettingreacties. Bijvoorbeeld, één eiwit kan een ander eiwit activeren en dat eiwit inactiveert op zijn beurt een derde eiwit. Deze kettingreacties, ofwel 'pathways', zijn belangrijk voor het functioneren van de cel en één fout in één van de eiwitten betrokken in de schakeling kan op vele niveaus zijn effect hebben. Daarbij komt nog dat de verschillende 'pathways' vaak invloed op elkaar uitoefenen en dat een verandering in de ene 'pathway' gevolgen kan hebben voor het functioneren van de andere. Kort gezegd, fouten in de genen leiden tot fouten in de eiwitten, welke op hun beurt het functioneren van de cel kunnen belemmeren.

Tijdens het leven delen de cellen keer op keer en bij elke celdeling worden de genen gekopieerd zodat elke dochtercel opnieuw het volledige pakket aan genetische informatie bevat. Hoewel het kopiëren van de genen een heel precies proces is, treden er bij elke deling honderden zo niet duizenden fouten op. Deze fouten worden vrijwel altijd gerepareerd. Er zijn verschillende controlemechanismen in de cellen die de fouten detecteren. Vaak kan de fout hersteld worden maar als dit niet mogelijk is gaat de cel dood door gereguleerde celdood (apoptose). Heel soms blijft echter een kopieerfout onopgemerkt. Als de betreffende cel opnieuw deelt zal ook de fout worden gekopieerd en zo ontstaat er langzaam een groepje cellen met allen een fout in de genen. Fouten kunnen natuurlijk ook ontstaan door kankerverwekkende omgevingsfactoren of voedingstoffen. Soms worden de fouten van de vader en/of moeder geerft en zijn ze al vanaf de geboorte aanwezig. Vooral als de fouten in de genen de cellen een groeivoordeel geven en/of de kans op een volgende fout vergroten gaat de groei van zo'n groepje cellen ten koste van de normale cellen. Deze genetische fouten staan aan de basis van het ontstaan van kanker.

Kwaadaardige, of maligne, tumoren ontstaan door een reeks fouten in de genen die aan volgende generaties cellen worden doorgegeven en niet worden hersteld maar zelfs tot een verhoogde kans leiden op nog meer fouten en de cellen een groeivoordeel geven t.o.v. hun normale tegenhangers. Het proces van normale lichaamscel naar maligne tumorcel wordt carcinogenese, tumorprogressie of tumorgenese genoemd. Tumorprogressie duurt vele jaren, is opgebouwd uit vele stappen en wordt veroorzaakt door een opeenstapeling van fouten in de genen, waardoor verschillende processen in de cel misgaan. Bij kanker zijn de meest in het oog springende celprocessen die verstoord worden de regulatie van de celdeling en apoptose bij kopieerfouten in de genetische informatie. Celdeling is

verhoogd en apoptosis is verlaagd. Maar ook andere processen in de cel moeten verstoord zijn voordat er sprake is van een maligne tumor.

In dit proefschrift zijn afwijkingen in genen en eiwitten in kanker van de alvleesklier, ofwel de pancreas bestudeerd. Hoewel er verschillende vormen van pancreas kanker bestaan ligt de focus op het ductale type pancreas tumoren: de pancreas adenocarcinomen (PA).

PA is een buitengewoon agressieve vorm van kanker. Patiënten presenteren zich meestal pas als de tumor zich lokaal heeft uitgebreid of wanneer uitzaaiingen op afstand aanwezig zijn. Hierdoor zijn de meeste tumoren (ongeveer 85%) niet meer te verwijderen, waardoor de gemiddelde overleving na diagnose slechts 5 maanden is. Elk jaar sterven er in de Verenigde Staten meer dan 37.000 patiënten aan pancreaskanker en hoewel er de laatste jaren veel tijd en geld is gestoken in onderzoek, zijn deze cijfers nauwelijks verbeterd. Ons begrip van de ontstaanswijze van pancreastumoren is enorm gegroeid in de laatste jaren, maar dit is nog niet vertaald in betere diagnostiek of behandeling. Op dit moment wordt gemcitabine, een vorm van chemotherapie, gezien als de beste behandeling na chirurgische verwijdering van de tumor, maar zelfs met de meest optimale behandelingsstrategie is de prognose van PA extreem slecht.

Wat als een paal boven water staat is dat PA, zoals alle kwaadaardige tumoren, een ziekte van de genen is. Veranderingen in gen en eiwitexpressie leiden tot veranderingen in het functioneren van de cel, met name in processen als celdeling, het aanzetten tot aanmaak van bloedvaten, apoptose, migratie en invasieve groei. Het bestuderen van genetische afwijkingen die een rol spelen bij de ontwikkeling van PA kan leiden tot de ontdekking van moleculen die kunnen dienen als aangrijpingspunt voor diagnostiek of behandeling. Daarnaast is het bestuderen van celprocessen betrokken bij prognose of overleving van de patiënt van belang omdat ingrijpen in deze verstoorde processen potentieel verbetering van de situatie van de patiënt kan geven.

Hoofdstuk 3 van dit proefschrift beschrijft de ontwikkeling van normale pancreas naar kwaadaardige PA zoals die momenteel gezien wordt. In de pancreas wordt de ontwikkeling van een kwaadaardige tumor voorafgegaan door pre-invasieve laesies, genaamd PanIN laesies. Deze PanIN laesies kunnen worden ingedeeld in verschillende stadia, gebaseerd op de morfologische en pathologische afwijkingen in deze PanIN. De verschillende PanIN stadia zijn gebaseerd op een specifieke combinatie van genetische afwijkingen, waaronder afwijkingen in oncogenen, tumor-suppressor genen en genen verantwoordelijk voor het behoud van de integriteit van het genoom. In de laatste 20 jaar heeft het onderzoek naar deze maligne progressie grote vooruitgang geboekt, onder andere dankzij de ontwikkeling van muismodellen voor PA waarin de genetische afwijkingen en de gevolgen daarvan voor het functioneren van de cel bestudeerd kunnen worden. De verschillende genetische defecten die worden geïnduceerd bij de muizen komen

overeen met de defecten gevonden bij patiënten met PA en weerspiegelen daardoor de tumorgenese in de mens op een betrouwbare manier. Op basis van onderzoek met deze muismodellen kan worden geconcludeerd dat een defect in het oncogen *KRAS2* de eerste stap is in tumorontwikkeling bij PA. Afwijkingen in de tumor-suppressor genen *CDKN2A* en *TP53* versnellen progressie naar maligne ontaarding.

In **hoofdstuk 4** ligt het focus op de genetische afwijkingen die worden gevonden in maligne tumoren van de pancreas. De aangedane genen coderen voor eiwitten die een rol spelen in verschillende 'pathways' en de genen worden in dit hoofdstuk besproken vanuit het perspectief van de functie die zij vervullen in de cel. Recentelijk werden 12 'pathways' geïdentificeerd die in bijna alle PAs afwijkingen vertoonden. Het doel van dit hoofdstuk was om deze 12 pathways te beschrijven in relatie tot hun functie in de cel. Wat geconcludeerd kan worden uit de genetische complexiteit van PA is dat het onwaarschijnlijk is dat er een behandeling kan worden ontwikkeld die bij iedere patiënt effect heeft. Het lijkt voor de hand te liggen dat het genetisch profiel van een individuele patiënt leidraad zal zijn voor het behandelplan. Maar momenteel is de kennis nog te beperkt hiervoor en zal er meer onderzoek gedaan moeten worden alvorens deze strategie kan worden toegepast.

Hoewel de gemiddelde overleving bij PA extreem kort is, is er een beperkt aantal patiënten die langer overleven dan verwacht. Waarom deze patiënten overleven terwijl andere patiënten overlijden wordt niet goed begrepen. Onderzoek naar prognostische variabelen of markers is daarom van groot belang. In **hoofdstuk 5** wordt hier een begin mee gemaakt. In de laatste jaren zijn vele onderzoeken gepubliceerd die prognostisch relevante karakteristieken van PA identificeerden. Vaak werd in de statistische evaluatie geen multivariate analyse verricht, de meest adekwate vorm van statistiek in dit soort onderzoek. Om te bepalen welke patiënt- en tumorkarakteristieken individueel van prognostische waarde zijn, werd een cohort samengesteld van zowel korte- als lange-termijn overlevers van PA. Een multivariate analyse werd uitgevoerd naar het effect op overleving van zowel klinische als morfologische en pathologische kenmerken. De studie toonde aan dat een lymfeklier ratio lager dan 0,2, tumorvrije snijvlakken bij operatie en expressie van het eiwit Smad4 onafhankelijke variabelen zijn die geassocieerd zijn met langere overleving.

In **hoofdstuk 6** wordt een ander onderzoek beschreven dat zich richt op de prognostische variabelen in PA. Voor dit onderzoek werd een uniek cohort samengesteld van lange-termijn overlevers van PA. Lange-termijn overleving werd gedefinieerd als meer dan 10 jaar overleving na diagnose. Uit de databases van 3 gespecialiseerde centra in de Verenigde Staten werd een groep van 24 lange-termijnoverlevers van PA samengesteld. Om te bepalen of de tumoren van deze patiënten op een vergelijkbare wijze waren ontstaan als tumoren van korte-termijn overlevende patiënten werd gescreend op afwijkingen in de meest voorkomende aangedane genen; *KRAS2* en *TP53*. Daarnaast werd een aantal eiwitten

geanalyseerd die werden verondersteld de prognose te beïnvloeden. De eiwitten Smad, dCK/hENT1 en microsatelliet instabiliteit werden geanalyseerd. Verassend genoeg toonde deze variabelen geen verschillen tussen de lange-termijn overlevers en de controlegroep van korte-termijn overlevers. Daarnaast werd er ook geen verschil gevonden in het percentage tumoren met fouten in de *KRAS2* en *TP53* genen tussen het lange-termijn overlevers cohort en de percentages gevonden in de literatuur. We concluderen hieruit dat de tumoren van de lange-termijn overlevers op een vergelijkbare manier ontstaan als de gemiddelde PA en dat er geen verklaring is voor de verlengde overlevingsduur.

Het hierboven beschreven cohort van >10-jaars overlevers met PA wordt in **hoofdstuk 7** verder onderzocht. Vijf van de lange-termijn overlevers werden geselecteerd voor kwantitatieve analyse om eiwitten te identificeren die een veranderd expressiepatroon vertonen in lange-termijn overlevers in vergelijking met een controlegroep bestaande uit 5 korte-termijn overlevers. Uit de analyse bleken verscheidene eiwitten een verschil in expressie te vertonen tussen de korte- en lange-termijn overlevers. Galectine-1, een eiwit dat betrokken is bij apoptose, werd geselecteerd voor verder onderzoek. Galectine-1 expressie werd bepaald in een cohort van PA patiënten met variabele overlevingstijd met behulp van immunohistochemie en dit toonde aan dat galectine-1 met name tot expressie komt in het stromale deel van de tumoren. Het stromale deel van tumoren bestaat uit niet-kankercellen die bindweefsel, bloedvaten en dergelijke vormen. Expressie van galectine-1 in het stroma bleek sterk geassocieerd te zijn met kortere overleving. Het exacte mechanisme van deze associatie is nog onbekend en verder onderzoek naar de rol van galectine-1 in PA is noodzakelijk. Daarnaast heeft de eiwit-expressie analyse andere indicaties opgeleverd welke in de toekomst verder zullen worden uitgediept.

Een van de eerder genoemde “pathways” die vaak is aangedaan in PA is de ”Notch pathway”. Deze “pathway” is van essentieel belang tijdens de embryonale ontwikkeling van de pancreas maar nadat het orgaan volledig is aangelegd worden de genen betrokken bij de “Notch pathway” inactief. Echter, in veel PAs blijken deze genen actief te coderen voor eiwitten. Op basis hiervan werd geconcludeerd dat remming van deze Notch genen een mogelijke behandelstrategie zou kunnen zijn. **Hoofdstuk 8** beschrijft de studie die met het voornoemde in gedachte werd opgezet. Met gebruik van een monoklonaal antilichaam gericht tegen een de Notch1 receptor werd onderzocht of het remmen van Notch activiteit invloed had op celdeling, overleving van de cel en celgroei in een milieu waar er geen communicatie tussen cellen mogelijk is. Er werden 2 PA cellijnen geselecteerd voor de experimenten. Behandeling van deze cellijnen met het antilichaam tegen de Notch1 receptor resulteerde in een duidelijke vermindering van celgroei in een geïsoleerd milieu. Echter, de behandeling had geen invloed op celdeling in groepen cellen of overleving van de cel. Hieruit kan geconcludeerd worden dat de “Notch pathway” met name invloed heeft op uitzaaiing omdat overleving in een geisoleerd milieu een van de voorwaarden hiervoor is. Verder onderzoek is nodig

om een exact beeld te krijgen van de rol die de verschillende componenten van de “Notch pathway” spelen in tumorgenese bij PA.

In 1997 werd gemcitabine geïntroduceerd als eerste keus behandeling na resectie van PA. Sindsdien zijn er verschillende andere behandelingsstrategieën onderzocht maar geen enkele andere behandeling bleek bij patiënten superieur te zijn aan gemcitabine. Hoewel *in vitro* vaak veelbelovende resultaten werden behaald, konden deze niet worden vertaald naar de kliniek. Recentelijk bleek FOLFIRINOX, een combinatie van de chemotherapieën 5-FU/folinezuur, irinotecan en oxaliplatin, de overleving bij uitgezaaide PA te verbeteren. Echter, gemcitabine is nog steeds eerste keus bij chirurgisch te verwijderen tumoren. **Hoofdstuk 9** beschrijft een studie naar het effect van MK-1775, een Wee1-remmer, bij behandeling van PA. WEE1 is een eiwit dat een ander eiwit activeert wanneer er fouten in de genen zitten die gerepareerd moeten worden voor celdeling. Het eiwit houdt de celdeling tegen zodat reparatie kan worden uitgevoerd. In tumoren met fouten in *TP53*, een tumor-suppressor gen, bleek MK-1775 in combinatie met gemcitabine een beter resultaat te geven dan behandeling met gemcitabine alleen. Eerder gepubliceerde studies hadden al aangetoond dat behandeling met MK-1775 leidt tot celdood omdat de cel nog niet de tijd heeft gehad de fouten in de genen te herstellen die nodig zijn voordat de cel in deling gaat. MK-1775 is bovendien gericht op het stromale deel van de tumoren en eerder is aangetoond dat behandeling met MK-1775 de concentratie van gemcitabine in de tumor verhoogd. Ook dit kan mogelijk bijdragen aan het effect van MK-1775 behandeling. Het verkleinen van de tumor zoals beschreven in dit hoofdstuk is in het verleden een goede indicator gebleken voor positieve resultaten in klinische studies en het lijkt dan ook juist om behandeling van PA patiënten met MK-1775 in combinatie met gemcitabine verder te onderzoeken.

Erfelijke vormen van kanker worden gekarakteriseerd door de aanwezigheid van kiembaan mutaties in oncogenen en tumor-suppressor genen. In veel gevallen zijn deze mutaties bekend en in het verleden heeft onderzoek naar de genetische basis van deze vormen van kanker geleidt tot een beter begrip van de niet-genetische tumoren in de aangedane organen. In **hoofdstuk 10** wordt een patiënt beschreven die eerder gediagnosticeerd was met een erfelijk maagkanker syndroom. Daarna werd bij hem een PA gevonden en de vraag rees of deze PA mogelijk veroorzaakt was door de *CDH-1* kiembaanmutatie die tot de erfelijke maagkanker had geleid. Na evaluatie van E-cadherine expressie, het eiwit product van *CDH-1*, kon worden geconcludeerd dat dit niet het geval was. Immunohistochemische evaluatie toonde een ongestoorde E-cadherine expressie in de PA en hieruit kon worden geconcludeerd dat de PA een sporadische tumor was die onafhankelijk van het erfelijke syndroom was ontstaan.

Hoewel de focus van dit proefschrift lag op de adenocarcinomen van de pancreas bestaan er ook andere soorten tumoren van de pancreas. In **hoofdstuk 11** wordt één van deze zeldzame varianten van maligne pancreastumoren beschreven, het

acinair celcarcinoom (ACC). Deze tumoren vormen een minderheid van 1-2% van alle kwaadaardige tumoren van de pancreas. Door deze lage incidentie is niet veel bekend van de ontstaanswijze. Recent werd een case report gepubliceerd welke een ACC beschreef met inactivatie van *LKB1* in een Peutz-Jeghers syndroom patiënt en hierdoor rees bij ons de vraag of *LKB1* een rol speelde in de tumorgenese van ACC. Van de 5 ACCs die werden onderzocht bleek geen enkele een defect te hebben in *LKB1*. Hieruit kon worden geconcludeerd dat *LKB1* geen rol speelt in de tumorgenese van ACC. Daarnaast kon de eerdere observatie dat ACC tumoren geen mutaties hebben in de genen die vaak aangedaan zijn in PA worden bevestigd. Meer onderzoek naar ACCs en de genetische afwijkingen die gepaard gaan met de ontwikkeling van ACC is nodig om een beter begrip te krijgen van deze zeldzame tumoren.

De onderzoeken beschreven in dit proefschrift tonen aan dat veranderingen in gen- en eiwitexpressie de drijvende kracht zijn achter het ontstaan van tumoren in de pancreas. Een goed begrip van deze veranderingen en hun relevantie voor het functioneren van de cel is de eerste stap in het verbeteren van de prognose en uitkomst van deze ziekte. De aangedane 'pathways' vertonen op een hoger niveau de effecten die individuele genetische defecten hebben op het functioneren van de cel en bij het ontwikkelen van nieuwe behandelingen is het belangrijk om deze in ogenschouw te nemen. De 'Notch pathway' is een goed voorbeeld hiervan hoewel er momenteel nog te weinig bekend is over de exacte rol van Notch activiteit in PA. Een andere mogelijk nieuwe invalshoek is om de behandeling te richten tegen het stromale deel van de tumoren. Het remmen van Wee1 in combinatie met gemcitabine lijkt op dit vlak de volgende stap.

Het is duidelijk dat er nog een lange weg te gaan is voordat tumorigenese van PA kan worden afgeremd. Maar er is wel veel duidelijk geworden omtrent het ontstaan van PA. Bij de behandeling van een ziekte zo complex en agressief als PA is het noodzakelijk om eerst een goed begrip te hebben van het ontstaan en de progressie van de ziekte alvorens nieuwe behandelingen kunnen worden gevonden. Dit is precies wat de laatste jaren heeft plaatsgevonden. De hoop is dat in de toekomst, deze kennis over de ziekte ontwikkeling zal leiden tot verbetering van de overleving van PA en hopelijk zullen de resultaten beschreven in dit proefschrift daaraan bijdragen.

About the author

Curriculum Vitae
List of publications

Curriculum vitae

Niki Anne Ottenhof was born on the eighteenth of may 1986 in Amsterdam, The Netherlands. In 1998, she started her secondary education at the Da Vinci College in Purmerend. She graduated in 2004 and in 2005 she started attending Medical School at the University of Amsterdam. She enrolled in the Honours program supervised by Prof. dr. C.J.F. van Noorden in addition to the standard curriculum. In 2009 she contacted Prof. dr. G.J.A. Offerhaus to explore the possibility of performing basic medical research. Professor Offerhaus provided her the unique opportunity to visit the Johns Hopkins University School of Medicine in Baltimore, Maryland. Supported by grants from the Dutch Cancer Society and the Dutch Digestive Foundation, Niki worked for ten months as a student research fellow at the department of Pathology in the Sol Goldman Pancreatic Cancer Research Center under supervision of Prof. dr. A. Maitra. Upon her return to the Netherlands, Niki continued her research at the department of Pathology at the University Medical Center Utrecht. In 2010 she started her clinical rotations for completion of her medical study while simultaneously working on completion of her thesis on “Molecular genetics of pancreatic carcinogenesis and their clinical significance”. In February 2011, Niki obtained her Master of Science degree in Medicine and started the last phase of the PhD trajectory. She will graduate from medical school in December 2012.

Niki was awarded the Nijbakker Morra Stichting Student Award for Cancer Research in 2011 for her work conducted at the Johns Hopkins University.

Ottenhof NA, Milne AN, Morsink FH, Drillenburg P, Ten Kate FJ, Maitra A, Offerhaus GJ. *Pancreatic intraepithelial neoplasia and pancreatic tumorigenesis: of mice and men.* Arch Pathol Lab Med. 2009;133(3):375-81

Bisht S, Mizuma M, Feldmann G, Ottenhof NA, Hong SM, Pramanik D, Chenna V, Karikari C, Sharma R, Goggins MG, Rudek MA, Ravi R, Maitra A, Maitra A. *Systemic administration of polymeric nanoparticle-encapsulated curcumin (NanoCurc) blocks tumor growth and metastases in preclinical models of pancreatic cancer*. Mol Cancer Ther. 2010;9(8):2255-64

Rajeshkumar NV, De Oliveira E, Ottenhof NA, Watters J, Brooks D, Demuth T, Shumway SD, Mizuarai S, Hirai H, Maitra A, Hidalgo M. *MK-1775, a potent Wee1 inhibitor, synergizes with gemcitabine to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts.* Clin Cancer Res. 2011;17(9):2799-806.

Ottenhof NA, de Wilde RF, Maitra A, Hruban RH, Offerhaus GJ. *Molecular characteristics of pancreatic ductal adenocarcinoma.* Patholog Res Int. 2011: 27;2011:620601

de Wilde RF, Ottenhof NA, Jansen M, Morsink FH, de Leng WW, Offerhaus GJ, Brosens LA. *Analysis of LKB1 mutations and other molecular alterations in pancreatic acinar cell carcinoma*. Mod Pathol. 2011;24(9):1229-36

Feldmann G, Mishra A, Bisht S, Karikari C, Garrido-Laguna I, Rasheed Z, Ottenhof NA, Dadon T, Alvarez H, Fendrich V, Rajeshkumar NV, Matsui W, Brossart P, Hidalgo M, Bannerji R, Maitra A, Nelkin BD. *Cyclin-dependent kinase inhibitor Dinaciclib (SCH727965) inhibits pancreatic cancer growth and progression in murine xenograft models.* Cancer Biol Ther. 2011;12(7):598-609

Ottenhof NA, de Wilde RF, Morsink FH, de Leng WW, Ausems MG, Morreau H, van Hillegersberg R, Offerhaus GJ, Milne AN. *Pancreatic ductal adenocarcinoma in hereditary diffuse gastric cancer. A case report.* Hum Pathol. 2012;43(3):457-61

Fendrich V, Oh E, Bang S, Karikari C, Ottenhof NA, Bisht S, Lauth M, Brossart P, Katsanis N, Maitra A, Feldmann G. *Ectopic overexpression of Sonic Hedgehog (Shh) induces stromal expansion and metaplasia in the adult murine pancreas.* Neoplasia. 2011;13(10):923-30

Ottenhof NA, Morsink FH, Ten Kate F, van Noorden CJ, Offerhaus GJ. *Multivariate analysis of immunohistochemical evaluation of protein expression in*

pancreatic ductal adenocarcinoma reveals prognostic significance for persistent Smad4 expression only. Cell Oncol (Dordr). 2012;35(2):119-26

Acknowledgements

Dankwoord

Acknowledgements

Toen ik in 2009 in het kantoor van professor Offerhaus plaatsnam, had ik geen enkel benul van het avontuur dat mij te wachten stond. Nu, 3,5 jaar later is het klaar. Om maar in cliches te vervallen: alleen was het niet gelukt. Velen hebben mij gesteund en geholpen en hiervoor ben ik iedereen buitengewoon dankbaar. Er zijn een aantal personen die ik in het bijzonder wil bedanken.

Ten eerste, prof. dr. Offerhaus, Johan, de meest onzelfzuchtige en hulpvaardige promotor die ik me voor kan stellen. Dankzij jou heb ik de kans gekregen dit project aan te gaan en heb ik mij mogen voegen in de wereld van het onderzoek. Zo groen als gras, maar jij nam mij onder je hoede en door de tijd heen heb ik altijd kunnen rekenen op je hulp en advies. Je bent een fantastisch wetenschapper maar een nog betere mentor.

Prof. dr. van Noorden, mijn 2de promotor, de man die al vanaf mijn tweede jaar geneeskunde een motivator was. Die mij leerde dat de sky the limit was als je het enthousiasme en de drive had om ergens voor te gaan. Ron, jij hebt een zeer grote rol gespeeld in mijn ontwikkeling tot de medicus en onderzoeker die ik ben geworden en ik ben zeer verheugd dat ik je heb mogen betrekken in het tot stand komen van dit proefschrift. Ik hoop dat er nog velen na mij zullen profiteren van jouw steun want deze is van onschatbare waarde.

Prof. dr. Anirban Maitra, my mentor in the formally unknown city of Baltimore and the world that is science. The first time we met I remembered something that Jan-Bart told. His advise was; when you are meeting with Anirban, have a piece of paper and a pen ready because when this guy starts talking, you are flooded with innovating ideas, plans and opportunities. And I was. Even without any prior accomplishments in the field of research, you accepted me as a full member of the team. I am extremely thankful for that.

Geachte leden van de commissie. Het is een eer dat jullie de tijd hebben genomen om mijn proefschrift te beoordelen en ik ben hier zeer dankbaar voor.

Furthermore, I am grateful to a lot of people from the Johns Hopkins University. First of all, prof. dr. Hruban, it was a great honor working with you. Thank you for the time you took out of your extremely busy schedule to work with me and help me with my projects. You are an extraordinary leader and I feel fortunate that I got to work with you.

My time in America wouldn't have been the same if it wasn't for Alexis. Alexis, you are an amazing person and I thank you for inviting me into your life and taking me on as a true friend. From the thanksgiving diner at your aunts house, black Friday shopping included, to throwing me a going away party. I am really grateful that we have stayed it touch and I'm looking forward to meeting you again this summer in Italy.

Hector, I really enjoyed the time we spent scoring IHC slides and I hope you found a nice place to practice medicine. Sonal, I know it was hard for you to deal with the unexperienced privileged Dutch girl but you kept your cool and taught me so much. Thank you for your time and energy. Collins, your 'good morning Niki' could really make my day. You are one of the funnies guys I know and you really helped me in achieving my goals. Savita and Georg, I was sorry to see you leave so soon after my arrival but you are a fantastic pair and I hope life in Germany is treating you well. Hanno, I really enjoyed working with you and talking about family and science. Seung, thank you for all the times you helped me score my IHC slides. You are a true master. Moreover, I would like to thank dr. Eshleman, dr. Iacobuzio-Danohue, Masa, Venu, Karen and Nathalie and all the other members from the lab.

Uiteraard iedereen in de Offerhaus groep, ontzettend veel dank voor alle hulp en adviezen. Jan-Bart, mijn voorganger. Ontzettend bedankt voor alle adviezen en hulp bij het volbrengen van dit project. Ik heb er heel veel aan gehad. Folkert, de immuno man! Dank voor het geduld waarmee je mij de eerste stapjes in het lab hebt leren maken, de adviezen op het IHC vlak, maar zeker ook de gezellige gesprekken over het thuisfront. Je bent van onschatbare waarde voor de groep Offerhaus. Wendy, dank voor je hulp en je enthousiasme. Altijd kon ik bij jou terecht voor vragen en adviezen en dankzij jou heb ik veel van mijn studies op een mooie volledige manier kunnen afmaken. Vicky, mijn theedrinkende wederhelft in het lab. Veel over onderzoek ging het niet, maar gezellig was het zeker. Ik geloof dat ik zonder onze koffie/theepauzes de laatste maanden niet door was gekomen dus ik ben je eigenlijk dit hele boek verschuldigd. De trip naar Polen was denk ik de kroon hierop, zonder jou gezelschap was het niet het zelfde geweest. Anya, dank voor de uitmuntende begeleiding bij het tot stand brengen van het E-cadherine stuk. Roeland, ik vond het erg fijn om met je te werken en ben trots op de stukken die we samen hebben weten te produceren. Succes met de afronding van jouw thesis! Verder wil ik graag bedanken Lodewijk, Daniëlle, Mirte en Marjon.

Mijn voltallige co-groepje, in het bijzonder Sophie, dank voor de afleiding van dit soms slopende proces. Jullie zijn stuk voor stuk toppers.

David, zoals beloofd, een bijzondere vermelding. Dank voor de adviezen maar natuurlijk met name dank voor het leveren van het ronduit onmisbare figuren-programma. Zonder jou zouden de grafieken er kinderlijk amateuristisch uit hebben gezien!

Lieve Em en Floor, mijn beste vrienden. Dank voor al de niet-wetenschappelijk gerelateerde steun. De avonden wijntjes drinken en praten. De hulp in deze niet altijd makkelijke tijd en de onvoorwaardelijke liefde die ik voel. Jullie zijn een constante factor in mijn leven en ronduit onmisbaar.

Lieve Koen, poerbroer, Tever Pegel, paranimf of hoe je het wil: heel veel dank voor je steun en liefde, zowel in Baltimore als in Nederland. Nog steeds kan ik hardop

lachen om de mailtjes die we hebben uitgewisseld. Jij hebt mij eloquentie geschonken in de geschreven taal, een groot goed. Maar meer nog dan dat, je bent het meest fantastische broertje dat een mens zich kan wensen.

Lieve Winnie, mijn liefste zus, zo niet dé liefste zus. Ik ben zo ontzettend blij dat ik jouw in mijn leven heb. Van het nietszeggend roddelen, het lachen om mijn soms ontspoorde gedachtegangen, het troosten als ik er echt doorheen zit tot de oprechte vreugde die ik bij jou voel als mij iets goeds overkomt. Je bent een van de belangrijkste personen in mijn leven en jouw rol in het goed doorkomen van de tijd in Amerika en daarna is onvoorstelbaar groot geweest.

Lieve Djaja, de intelligentste man die ik ken, mijn grote broer en mijn voorbeeld. We zien elkaar te weinig, maar tijdens onze ontmoetingen voel ik altijd je oprechte interesse in mijn leven en de dingen die spelen. Dat jij met Koen naar Amerika kwam om te zien hoe mij leven daar was heeft ontzettend veel voor me betekent en ik voel me vereerd dat je mijn paranimf wilde zijn. Veel geluk in de komende jaren met je kleine freubel.

En dan als laatste, papa en mama. Ik vond het niet makkelijk en ik heb het jullie niet makkelijk gemaakt. De tijd in Amerika was volgens mij een enorme uitdaging voor jullie en ik heb het met mijn numb hoofdje niet eens beseft. Het geduld waarmee jullie hebben geacteerd, de steun, de liefde. Jullie laten ons onze eigen fouten maken maar vinden het niet erg om daarna de stukken op te rapen. Nooit voel ik afstand of terughoudendheid om jullie in mijn leven te betrekken en ik hoop later op een zelfde manier mijn kinderen op te voeden. Dit boekje is door maar zeker ook voor jullie.