Clinical significance of molecular markers in pancreatic cancer
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Citation for published version (APA):

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Chapter 1

General Introduction and Outline of the Thesis
GENERAL INTRODUCTION:

Ductal adenocarcinoma of the pancreas is among the leading causes of cancer-related death in the Western countries. The incidence of pancreatic carcinoma in Europe is low, 10-15 per 100,000. Although pancreatic cancer accounts for only about 2% of all malignant tumors, it nevertheless represents the fifth most common cause of death due to cancer. The overall 5-year survival of pancreatic adenocarcinoma is very poor, approximately 2-5%. The dismal prognosis is largely a reflection of the late diagnosis in combination with the aggressive growth of the disease. For pancreatic cancer patients the only option for cure is radical resection of the tumor, but more than 85% of patients have a carcinoma extending beyond the pancreas at the time of diagnosis, and only 10 to 20% of patients have lesions that are resectable.

The diagnosis is partly hampered by the lack of specific symptoms attributable to early malignant disease. Patients generally show non-specific symptoms, such as anorexia, weight loss, and nausea, and more specific symptoms are jaundice, pruritus, pancreatic exocrine and endocrine dysfunction and back pain. However, these symptoms can also be caused by benign diseases in the pancreatic head region. In case of a clinical suspicion of a periampullary malignancy, ultrasonography with Doppler flow measurements and (intravenous contrast enhanced) spiral CT scan are generally used to exclude stone disease, detect a mass in that region, determine the presence and level of bile duct obstruction, visualize liver metastases (larger than 1-2 cm), determine the involvement of the major vessels, and detect presence of ascites (a sign of peritoneal metastases).

Another useful technique to visualize the presence and the level of bile duct stenosis is endoscopic retrograde cholangiopancreatography (ERCP). In some cases a cytological or histological confirmation of the cause of the disease is indicated, notably when this knowledge will affect treatment. In these cases a fine needle biopsy (FNA) or brushings of the distal common bile duct can be performed. Although these techniques are highly specific, the sensitivity remains low; for brush cytology obtained during ERCP the sensitivity is only 30-40%. The relatively low sensitivity of the ERCP brush cytology is partly a result of the low yield of malignant cells during this procedure.
Tumor progression
Morphologic and molecular studies have demonstrated that pancreatic cancer is the ultimate result of a stepwise progression from normal cuboidal duct epithelium via flat duct lesions without atypia (PanIN-1A), papillary duct lesions without atypia (PanIN-1B), papillary duct lesions with atypia (PanIN-2), to carcinoma in situ (PanIN-3), and finally invasive ductal carcinoma 14-16. PanIN stands for pancreatic intraepithelial neoplasia. These consecutive histological precursor stages are associated with an accumulation of specific and generalized molecular genetic alterations affecting a variety of cancer-causing genes 17-24 (figure 1). Because these genetic alterations occur when the neoplasm is still non-invasive, and before it has spread beyond the pancreas, these molecular changes can potentially be utilized in a variety of clinical applications, including screening of patients at risk, improvement of diagnosis, optimalization of staging procedures, characterization of the genetic make up of unusual pancreatic neoplasms, and prognostication of tumors with distinctive molecular features.

Genes involved in tumor progression of pancreatic carcinogenesis

Oncogenes
Genes with a function in the growth of normal cells, so-called proto-oncogenes, can become activated by point mutation, by gene amplification, or by fusion to other genes and their regulatory elements, and will then act as oncogenes. In pancreatic cancer the most intensively studied oncogene is the K-ras oncogene.

The K-ras oncogene
The K-ras oncogene is mutationally activated in 80-90% of pancreatic carcinomas 25, 26. By far the most mutations detected in pancreatic cancer occur in codon 12 of the K-ras oncogene. They typically involve a single base pair change of the GGT sequence (glycine) and sensitive polymerase chain reaction (PCR)-based assays can detect these mutations easily and reliably 26-28.
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*K-ras* codon 12 mutations are a very early event in the pancreatic tumor progression model. In fact, it has been shown that the *K-ras* oncogene can already be mutated in pancreatic duct epithelium that microscopically appears normal 19, 29-32. The high incidence and early occurrence of *K-ras* alterations suggest that *K-ras* mutations might serve as a qualitative marker for this disease, but at the same time indicate that specificity could be a concern. Therefore, many studies have focussed on the detection of *K-ras* mutations in secondary sources, including fine-needle aspirates (FNA), ERCP brush cytology, pancreatic juice, bile, duodenal aspirates, blood and stool samples 27, 28, 33-37.

*HER-2/neu*

*HER-2/neu* is a member of the epidermal growth factor receptor family 38. Between 20-70% of pancreatic carcinomas show overexpression of *HER-2/neu* 17, 38-40. Overexpression of *HER-2/neu* is also considered as an early genetic event in pancreatic carcinogenesis. *HER-2/neu* is virtually not expressed in normal pancreatic duct epithelium, whereas 82% of the flat duct lesions (PanIN-1a en 1b) and 92% of the atypical papillary duct lesions (PanIN-2) show expression 17. It is not well established what causes that the gene is overexpressed, but unlike breast carcinoma gene amplification does not seem to be the underlying mechanism 40.

**Tumor suppressor genes**

Tumor suppressor genes normally suppress cell growth by multiple mechanisms, including cell cycle control, transcription regulation and induction of apoptosis. Whereas an activating mutation in one of the two copies of an oncogene is sufficient for their oncogenic activity, in contrast, inactivating mutations of both alleles of a tumor suppressor gene are needed to disrupt their normal suppression of the cell growth. The tumor suppressor gene that mostly is inactivated in pancreatic cancer is the *MTS-1* (multiple tumor suppressor gene) or *p16* gene on chromosome 9p.

**The *p16* gene**

The *p16/MTS-1/INK4a* tumor suppressor gene is an inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6. CDK4 in combination with cyclin D1 phosphorylates the retinoblastoma
gene Rb, after which the cell can proceed through the G1 phase to the S-phase. The p16/Rb pathway is inactivated in virtually 100% of pancreatic tumors studied. Mechanisms by which the p16 gene is inactivated include homozygous deletion (40%), intragenic mutation coupled with loss of the wild type allele (40%), and in approximately 15% of the cases the p16 gene is inactivated by hypermethylation of its promoter region. Germline mutations in the p16 gene cause the familial atypical multiple mole melanoma (FAMMM) syndrome, and patients carrying this germline mutation have an increased risk for both melanoma and pancreatic cancer.

Inactivation of the p16 tumor suppressor gene appears to occur slightly later than alterations in the K-ras and HER-2/neu oncogenes.

The p53 gene

The p53 tumor suppressor gene is a transcription factor activated in response to DNA damage. Activation of p53 can induce apoptosis and G1 cell cycle arrest. Moreover, the p53 gene is directly involved in DNA repair itself. Thus, inactivation of the p53 tumor suppressor gene leads to the loss of three important cell cycle controls. The gene is inactivated in 50-70% of pancreatic carcinomas and it is believed to be a relatively late event in pancreatic carcinogenesis. Mutations of the p53 gene are typically found at the stage of carcinoma in situ or invasive carcinoma. The detection of p53 mutations is facilitated by the prolonged half-life of the mutant protein due to conformational changes of the protein product caused by most of the mutations. Although not a perfect surrogate for mutational sequence analysis, immunohistochemistry can detect mutated and therefore accumulated p53 protein with a sensitivity of 65-70% and 90% specificity.

The DPC4 gene

The DPC4 tumor-suppressor gene is located on chromosome 18q and is inactivated in approximately 55% of infiltrating pancreatic carcinomas. Dpc4 is a phosphoprotein with sequence-specific DNA binding properties and it is able to form complexes with various phosphorylated Smad proteins. These activated complexes are translocated to the nucleus where their binding to DNA stimulates the transcription of genes (figure 2). Germline DPC4
gene mutations cause juvenile polyposis. Loss of DPC4 expression is a biologically late event in the pancreatic progression model and is only encountered at the stage of carcinoma \textit{in situ} or invasive carcinoma. Using a sensitive and specific antibody against Dpc4 protein it was shown that DPC4 expression was lost in 9 of 29 (31%) PanIN-3 lesions, but was present in all of the 159 lower grade PanINs (PanIN-1 and PanIN-2) \cite{23, 55}.

\textbf{The BRCA2 gene}

The \textit{BRCA2} gene is thought to prevent DNA strand breaks that occur during normal cell division \cite{58, 59}. A potentially significant therapeutic consequence of disrupted BRCA2 function is the associated increased radio-sensitivity of BRCA2 deficient cells that has been observed in vitro and in animal models \cite{58, 59}.

It is estimated that approximately 5-10\% of patients with clinically sporadic pancreatic cancer harbor germline mutations of the \textit{BRCA2} gene \cite{60, 61}. Patients with a germline mutation in the \textit{BRCA2} gene have an increased lifetime risk of breast, ovarian and pancreatic cancer \cite{60-63}. The prevalence of biallelic inactivation of the \textit{BRCA2} gene in precursor lesions of patients with a germline mutation who developed pancreatic cancer has been investigated. Biallelic inactivation was observed in one high-grade PanIN (PanIN 3), but in none of the 13 low-grade PanIN lesions (PanIN 1), suggesting that \textit{BRCA2} gene inactivation is a late event in pancreatic carcinogenesis \cite{21}.

\textbf{The STK11/LKB1 gene}

Germline mutations in the \textit{STK11/LKB1} gene are the cause of the Peutz-Jeghers syndrome (PJS), which is an autosomal dominant condition characterized by multiple gastrointestinal hamartomatous polyps and the presence of pigmented lesions on the lips and oral mucosa \cite{64}. Patients with Peutz-Jeghers syndrome have an increased risk for developing pancreatic cancer \cite{65}. The \textit{STK11/LKB1} gene is somatically inactivated in 4\% of sporadic pancreatic cancers. Of 53 PJS patients analyzed in four independent studies, 11\% developed pancreatic adenocarcinoma \cite{66}.

\textbf{Other genes}

Other genes that are responsible for familial syndromes associated with increased risk of pancreatic cancer include the cationic trypsinogen gene, \textit{PRSS1}, responsible for hereditary
pancreatitis. Carriers of a germline mutation in this gene have a lifetime risk of pancreatic cancer approaching 50%\(^67, 68\).

The mitogen-activated protein kinase (MAPK) kinase 4 (\(MKK4\)) gene encodes a protein which is a component of a stress and cytokine-induced signal transduction pathway involving MAPK proteins. \(MKK4\) is a target of inactivation in a small percentage of pancreatic cancers\(^69\).

The TGF\(\beta\) type I receptor gene, \(ALK-5\), was homozygously deleted in 1 out of 97 pancreatic carcinomas. Somatic alterations of the TGF\(\beta\) type II receptor gene were found in 4 out of 97 (4.1\%) pancreatic carcinomas\(^70\).

**Mismatch repair genes**

Six human mismatch repair genes have been identified to date and they include \(hMSH2\), \(hMLH1\), \(hPMS1\), \(hPMS2\), \(hMSH6/GTBP\) and \(hMSH3\). Once a mismatch repair gene is inactivated, de novo mutations are not repaired properly, and this results in increased genetic instability, and a basis on which carcinomas can evolve relatively rapidly\(^71, 72\).

Approximately 4\% of pancreatic cancers show microsatellite instability and those tumors have a different clinicopathological phenotype than conventional pancreatic adenocarcinomas, with similarities to colorectal carcinomas showing microsatellite instability, including a medullary-type growth pattern, a better prognosis and a distinct mutational spectrum\(^73-75\).

**Risk factors**

For prevention purposes it is crucial to focus on the identification of individuals at-risk and to detect cancers arising in such individuals at an early disease stage. Several risk factors for pancreatic carcinoma have been described.

Pancreatic cancer is rare before the age of 40 and the majority of cases occur between age 60 and 80 yrs and slightly more often in men than in women\(^1\). Cigarette smoking is strongly related to pancreatic cancer: smokers have about a 2- to 3-fold increased risk of pancreatic cancer, and as is the case in lung cancer, the risk of pancreatic cancer increases with the number of pack-years of exposure\(^76, 77\). Also dietary factors have been associated with the development of pancreatic cancer. A high intake of fat and meat and a low intake of fiber are
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associated with an increased pancreatic cancer risk, whereas a diet rich in fruit and vegetables appears to be protective 78-81. Furthermore, chronic pancreatitis causes about a 15-fold increased risk of pancreatic cancer 82. For patients with hereditary pancreatitis, a rare familial form of pancreatitis caused by germline mutations in the cationic trypsinogen gene, *PRSS1*, located at chromosome 7q35, the estimated life-time risk of pancreatic cancer is about 50% 67, 68.

Several other genetic disorders are also accompanied by an increased risk of pancreatic cancer, such as hereditary non-polyposis colon cancer (HNPCC) syndrome, caused by a germline mutation in one of the DNA mismatch repair genes 83, and the familial adenomatous polyposis syndrome (FAP), caused by a germline mutation in the adenomatous polyposis coli (*APC*) gene 84.

In some studies diabetes emerged as a risk factor for pancreatic cancer, but the relationship is uncertain, since diabetes can be an early symptom in the development of pancreatic cancer 85, 86.

Furthermore, patients who underwent peptic ulcer surgery appear at higher risk for developing subsequent pancreatic cancer, especially after a prolonged postoperative interval 80, 87, 88.
Figures 1 and 2.

**Figure 1.** Progression model for pancreatic cancer. The progression from histologically normal epithelium to low-grade PanIN to high-grade PanIN (left to right) is associated with the accumulation of specific genetic alterations.

**Figure 2.** Upon binding to one of the ligands, the TGF-β receptor I (R1) is sequestered into the complex and is phosphorylated by TGF-β receptor II (R2). Subsequently, R1 sends downstream signals for gene activation (through phosphorylation events) that directly phosphorylates SMAD2, which forms a complex with DPC4/SMAD4. The complex enters the nucleus and acts as a transcriptional activator of genes involved in TGF-β function, such as growth inhibition.
REFERENCES:


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Introduction


Chapter 1

OUTLINE OF THE THESIS

As mentioned earlier, many genetic alterations are required for the development pancreatic cancer. Bringing the knowledge of cancer genetics into clinical practice may have implications for screening, prognosis and therapeutic decision making. Identification of patients at increased risk for pancreatic carcinoma, assessment of molecular markers for early detection and more accurate diagnosis, and investigation of the utility of molecular genetic tests for staging and prognostication is therefore of utmost importance.

The identification of individuals at increased risk for pancreatic cancer is essential for (future) surveillance programs. For example, family members of known hereditary syndromes, such as the familial breast cancer syndrome due to a germline BRCA2 mutation and patients with hereditary pancreatitis due to PRSS1 germline mutation, carry a higher risk for pancreatic cancer than the general population and could be candidates for screening. In chapter 2 a review is presented on the familial segregation of pancreatic carcinoma.

Postgastrectomy patients may constitute another risk group for pancreatic carcinoma and therefore a population with an increased index of suspicion. In chapter 3 the long-term risk of pancreatic cancer development was assessed in a cohort of patients who underwent a gastrectomy for benign peptic ulcer disease.

In chapter 4 the literature is reviewed with regard to the role of molecular-based diagnostic tests applied to sources other than pancreatic tissue itself, including ERCP-samples, blood and stool, with emphasis on the detection of K-ras mutations and mutant p53 gene product. In chapter 5 the possible diagnostic use of p53 immunocytochemistry in ERCP brush cytology specimens is evaluated.

In chapters 6 and 7 the diagnostic and prognostic value of DPC4 immunohistochemistry is evaluated. In chapter 8 a rare form of pancreatic cancer, pancreatic mucinous cystic neoplasms with sarcomatous stroma, is described and the question regarding the clonal origin of the two components in this tumor is addressed by means of molecular analysis. Chapter 9 provides a summary and general discussion of the main findings of these studies.