Prognostic Markers in Pancreatic Cancer:

the Tumour and its Environment

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Prognostic Markers in Pancreatic Cancer:

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de tumor en zijn omgeving

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Introduction and outline thesis

Incidence

Pancreatic cancer is not one of the most common types of cancer; however it is most certainly one of the most devastating types, ranking fourth in the list of cancer related deaths with a 5-year survival of only 6%. In 2010 there were an estimated 43.140 new cases whereas 36.800 patients were expected to die from this disease in the United States.[1] Worldwide, the expected numbers were 278.684 new cases and 266.669 deaths.[2] In the Netherlands 2481 patients died of pancreatic cancer in 2010.[3] Interestingly, whereas for most cancers, death rates have decreased over the years, those of pancreatic cancer have remained relatively stable.[1]

Pathogenesis

In order to effectively diagnose, prevent and treat pancreatic cancer, a detailed understanding of the molecular biology of this disease is required. Like many other cancers, pancreatic cancer results from the accumulation of genetic alterations. It originates in the ductal epithelium and evolves from non-invasive precursor lesions of which pancreatic intraepithelial neoplasias (PanINs) are the best characterized. The progression from minimally dysplastic epithelium (PanIN grade 1) to more severe dysplasia (PanIN grades 2 and 3) and finally to invasive carcinoma is paralleled by the successive accumulation of genetic alterations of which some appear earlier than others (figure 1).[4]

A brief overview of the molecular mechanisms underlying the biology of pancreatic cancer is given in chapter 2.

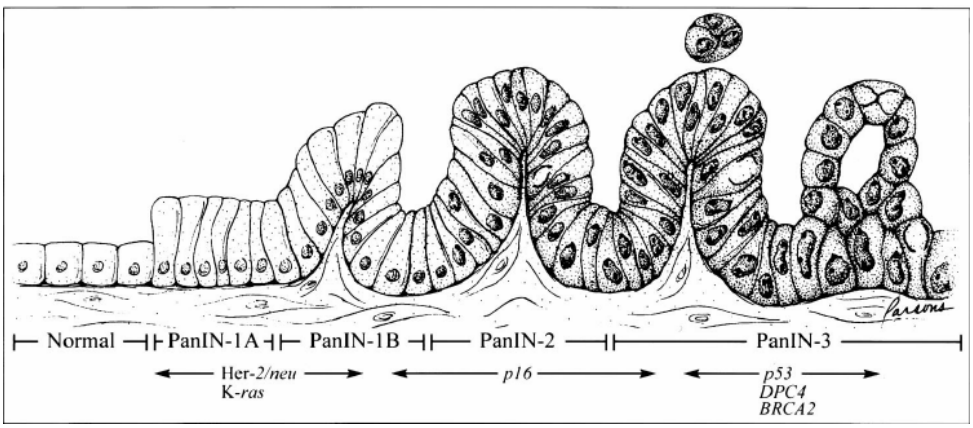

Fig. 1 – Progression model for pancreatic cancer. Normal duct epithelium progresses to infiltrating cancer (left to right) through a series of histologically defined precursors (PanINs). The overexpression of HER-2/neu and point mutations in the K-ras gene occur early, inactivation of the p16 gene at an intermediate stage, and the inactivation of p53, DPC4, and BRCA2 occur relatively late. (Reprinted with permission from AACR)

Diagnosis, staging and treatment

Currently, surgical resection is the only option for cure of pancreatic cancer. However, most patients present with advanced disease precluding them from resection. Resectability is generally determined by the UICC or AJCC TNM staging system. Based on pre-operative contrast enhanced multi-slice CT or MRI images, patients can be classified as having localized, resectable (stage I and II, 7% of cases), locally advanced (stage III, 26% of cases) or metastatic (stage IV, 53% of cases) pancreatic cancer.[1,5] With the progress in radiological imaging and surgical techniques, a different subset of stage III tumours can be defined; the borderline resectable tumours. These include those tumours with abutment of ≤180° of the circumference of the superior mesenteric artery; short encasement of the celiac axis or common hepatic artery; short segment occlusion of superior mesenteric or portal veins with a technical option for reconstruction.[6]

Pre-operatively tumours of the distal common bile duct and those of the ampulla of Vater that extend into the pancreas are often difficult to differentiate from those arising in the head of the pancreas, especially in case of large tumours. Therefore no difference is made in the surgical treatment of these cancers. However post-operative survival of periampullary cancer is significantly better than that of pancreatic head cancer.[7,8] We therefore decided to differentiate between both tumours in our studies.

Adjuvant treatment

Even following resection prognosis remains poor, with at the best only 20% being alive 5 years following surgery.[9] Therefore several adjuvant treatment regimens have been investigated. The most widely investigated chemotherapeutic agent in the treatment of pancreatic cancer is the fluoropyrimidine 5-fluoracil (5-FU). The first randomised trial comparing 5-FU based chemoradiotherapy (CRT) to surgery alone was the GITSG 9173 trial. This study identified a significant survival benefit for the adjuvant treatment group.[10] This relatively small study was followed by the EORTC trial comparing postoperative combined infusional 5-FU and radiotherapy given in a split course with observation only in patients with resected pancreatic and periampullary cancer. In this study only a trend for a benefit was observed for the CRT arm.[11] Interestingly, in the ESPAC-1, a significant benefit of the chemotherapy only arm over the observation arm was observed, whereas CRT showed to have a deleterious effect on survival.[12] This study was the beginning of a continental divide in the treatment of pancreatic cancer between Europe and the United States of America (USA). Chemotherapy alone is mostly used in Europe, whereas CRT is standard of care in the USA.

Another chemotherapeutic agent, tested in the adjuvant setting after its success in the palliative setting, was the nucleoside analogue gemcitabine. In the CONKO-001 study gemcitabine was compared with observation following resection of pancreatic cancer. In this study a significant disease-free survival benefit for the gemcitabine arm was found, however for overall survival only a trend for a benefit was observed.[13] The RTOG 9704 compared CRT with gemcitabine to CRT

alone and found no significant differences in survival.[14] The most recent study, and largest to date, is the ESPAC-3, comparing bolus 5-FU/leucovorin with gemcitabine following surgery. Survival proved similar in both arms, suggesting that gemcitabine is not superior to 5-FU.[15,16]
Another randomised controlled study was performed at our own institution, comparing combination celiac artery infusion (5-FU, mitoxantrone, leucovorin and cisplatinum) and radiotherapy, with surgery alone. No significant difference in survival was observed between the groups.[17]
The studies described in chapter 3 to 8 include patients from both this institutional study and the EORTC trial conducted in Rotterdam.

Although some of these trials suggest prolonged survival compared to their control groups, outcome is not significantly different from prior reports showing a 18-20 months median survival with surgery alone.[18] Two meta-analysis conduc-ted, one evaluating the role of adjuvant chemotherapy for patients with resected pancreatic cancer, the other the benefit of 5-FU based adjuvant CRT, had to conclude that the significance of adjuvant therapy remains unproven in the context of current diagnostics and surgical practice, and the optimal regimen still remains to be defined.[19,20]

The limitations of current treatment protocols for pancreatic cancer warrant an alternative approach. The problem of most therapeutic agents is that they generally only benefit a subset of treated patients. The delineation of cancer phenotypes based on molecular markers of therapeutic responsiveness and overall outcome can enable stratification of patients to appropriate individualized therapeutic regimens, so that optimal treatment is given without delay and unnecessary adverse side-effects are minimized. Several proteins have been investigated as prognostic markers in pancreatic cancer; however none have been implicated in clinical practice yet.[21-25]

Resection margin

Although evaluation of the resection margin is not part of the TNM staging system, it is of paramount importance for the prognosis of pancreatic cancer. Survival data suggest that survival of patients with incomplete resections (R1 for microscopically incomplete and R2 for macroscopically incomplete) is no different from that of patients with locally advanced, surgically unresectable disease treated with chemoradiation.[5,6] Including these R1 patients in prognostic biomarker studies would negatively influence interpretational value; we therefore only included complete (R0) resections in our prognostic studies.

Tumour micro-environment

A characteristic of pancreatic cancer is the formation of dense stroma, termed desmoplastic reaction. Traditionally research on cancer focussed on the autonomous properties of the tumour cell, neglecting the tumour micro-environment. It is

now apparent though, that the cells constituting the tumour stroma are not merely passive bystanders, rather they are critically involved in the process of tumour formation, progression, invasion and metastasis.[26-29]

The general aim of this PhD thesis was to identify prognostic markers following radical (R0) resection of pancreatic head or periampullary cancer that could potentially aid in therapeutic decision making. In doing so we focused on both tumour characteristics and the tumour micro-environment.
In chapter 3 the expression and prognostic value of major target of 5-FU and rate-limiting enzyme in the novo synthesis of DNA, thymidylate synthase (TS), was evaluated.
Apart from sustaining growth abilities, cancer cells have the ability to evade cell death, apoptosis. A key player in apoptosis is the Bcl-2 family of proteins. Chapter 4 describes the expression and prognostic value of a novel Bcl-2 asssociated anthanogen, Bag-1.
It is believed that angiogenesis is a prerequisite for a tumour to grow beyond a certain size and metastasize to distant sites. In chapter 5 we therefore explored tumour vascularity and its role in the prognosis of pancreatic cancer.
Pancreatic cancer prognosis is mainly determined by its metastasis. The basement membrane (BM) constitutes the first natural barrier to invasion and the formation of metastases. Widely fragmented basement membranes have been observed in pancreatic cancer amongst a range of cancers. In chapter 6 basement membrane delineation by its major components, laminin and collagen type IV was evaluated for its association with outcome of pancreatic head and periampullary cancer.
Another molecule, HMGA1, involved in invasion and metastases formation and present in cancer cells whereas absent in their normal counterparts, was evaluated for its prognostic value in chapter 7.
To conclude, chapter 8 comprises a systematic review of prognostic markers in radically resected pancreatic- and periampullary cancer.

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Abstract

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. It is a highly aggressive malignancy for which currently available treatments are of only limited efficacy. For this reason, much research is directed at elucidating fundamental molecular mechanisms underlying the biology of pancreatic cancer. These efforts are generating a rapidly growing body of information. The yet unmet challenge is to translate this information into clinically applicable strategies for early detection, prediction of prognosis, and effective therapies for patients diagnosed with pancreatic cancer.

An estimated 33,730 patients will be diagnosed with pancreatic cancer this year in the United States.[1] As a result of multiple factors, including the aggressiveness of this cancer and lack of effective screening strategies, most patients are diagnosed with locally advanced or metastatic disease, for which currently available treatments are of limited efficacy.[2-7] As a result, pancreatic cancer will cause more than 32,300 deaths in the United States in 2006, making it the fourth leading cause of cancer-related death in both men and women in this country.[1,8,9]
Since initial recognition of the importance of K-ras mutations in pancreatic cancer during the late 1980s, understanding of the biologic mechanisms underlying the behavior of this cancer has been growing at an increasingly rapid pace. There is reason to hope that this information will ultimately lead to the development of (1) improved diagnostic strategies that will allow for detection of premalignant (and therefore curable) lesions, and (2) targeted therapies for patients diagnosed with pancreatic cancer. In this brief review, we present an overview of our current understanding of the molecular biology of pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer.

Oncogenes and tumor tuppressor genes

Oncogenes arise as the result of mutations in normal genes (called proto-oncogenes) that regulate processes such as cell cycle progression.[5] As a result of these mutations, the protein products normally encoded by these genes are altered in a way that results in new or increased activity within the cell. In contrast, tumor suppressor genes encode proteins that inhibit processes such as cell proliferation. Mutation and/or deletion of tumor suppressor genes eliminates these inhibitory functions.[10] The consequences of these two types of gene alterations allow a cell to acquire features of the malignant phenotype (eg, increased proliferation, ability to evade apoptosis, and the capability for invasion and metastasis). Below we describe four oncogenes and tumor suppressor genes that, because of their prevalence and central roles in pathogenesis of PDAC, constitute the genetic signature of this cancer. Table 1 lists these genes and their corresponding chromosomal location, lesion type, and estimated frequency.

K-ras

Although there is variability in the literature regarding the true prevalence of activating mutations in K-ras in PDAC, studies of resected tumors suggest that this mutation is present in nearly all cases.[5,11–14] Indeed, K-ras mutation is widely believed to be one of the earliest, and possibly critical, events in the pathogenesis of PDAC.[5,12] Located on chromosome 12, the K-ras gene encodes a member of the Ras family of GTP-binding proteins that transduces cellular growth, differentiation, and survival signals.[14,15] Point mutations, occurring principally at codon 12 in the K-ras gene, impair the protein's intrinsic GTPase activity, thereby causing it to be-

come locked in its active (GTP-bound) form.[5,11,16] The downstream consequences of constitutively active K-ras signaling are discussed later.

p16 (CDKN2, p16INK4a, MTS1)

The p16 tumor suppressor gene is inactivated in approximately 95% of pancreatic carcinomas.[2,14,17] The gene is located on chromosome 9, where it encodes a protein that inhibits entry into the S phase of the cell cycle by inhibiting cyclin-dependent kinase (CDK) 4/6-dependent phosphorylation of retinoblastoma (RB) protein. The consequence of p16 inactivation is unregulated cell growth by inappropriate progression through the cell cycle.[4,5,13,16] Mechanisms of p16 inactivation include homozygous deletion, intragenic mutation plus loss of heterozygosity (LOH), and promoter hypermethylation.

p53

The p53 tumor suppressor gene is inactivated in 50% to 75% of PDACs.[2,4,14] The gene is located on chromosome 17, where it encodes a transcription factor that regulates the expression of a range of genes important in cell-cycle progression, apoptosis, and DNA repair. Among the important functions of the p53 product is inhibition of cell-cycle progression in the face of DNA damage; a consequence of p53 inactivation is loss of cell-cycle "check-point" function.[13,16] The mechanism of p53 inactivation is intragenic mutation, resulting in a defective product unable to bind DNA.

Gene Deleted in Pancreatic Carcinoma, Locus 4 (DPC4/SMAD4)

The Deleted in Pancreatic Cancer, locus 4 (DPC4) gene is inactivated in 55% of pancreatic cancers.[18] The gene is located on chromosome 18, where it encodes a protein that plays a key role in the transforming growth factor-beta (TGF-β)–mediated growth inhibitory signal transduction pathway.[14] DPC4 inactivation may result in dysregulated progression through the cell cycle.[13] Mechanisms of DPC4 inactivation include homozygous deletion and intragenic mutation plus LOH.

Table 1 - Prevalent genetic lesions in pancreatic ductal adenocarcinoma.

Gene	Location	Lesion	Estimated Frequency
K-ras	12p	Activating mutation	75–100%
P16	9p	Loss	~95%
P53	17p	Inactivating mutation	50–75%
DPC4/SMAD4	18q	Loss	~55%

Growth factors and their receptors

Relative to normal pancreatic tissues, PDACs overexpress a wide range of growth factors and their receptors. Downstream signaling mediated by growth factor li-

gand-receptor interactions are likely to play important roles in a range of phenotypic features of PDAC, including growth, invasion, and angiogenesis. Notable examples are described below.

Epidermal Growth Factor

Epidermal growth factor receptors (EGFR) are membrane receptor tyrosine kinases that mediate cellular proliferation and survival signals.[16] EGFR is overexpressed in PDAC, and overexpression of the EGFR and one or more of its ligands appears to be a marker of poor prognosis in patients with PDAC.[6,7,16,19,20] Antibodies directed against EGFR (eg, cetuximab) and inhibitors of its tyrosine kinase activity (eg, erlotinib) are currently undergoing evaluation in the treatment of PDAC.[19–23]

Transforming Growth Factor-β

The TGF-β family of proteins is associated with a complex array of functions, notably inhibition of cellular proliferation. Inactivation of DPC4/SMAD4 in pancreatic cancer cells may allow them to escape the growth inhibitory effects of TGF-β.[24] Postulated promalignant effects of TGF-β signaling include promotion of invasion and angiogenesis.[25]

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) promotes endothelial cell proliferation and survival and, hence, promotes angiogenesis. VEGF is overexpressed by pancreatic cancer cells and in pancreatic cancer tissues.[16] A monoclonal antibody directed against the VEGF receptor (bevacizumab) is currently being evaluated in clinical trials of patients with pancreatic cancer.[20,21,23,26]

Signaling cascades

Information on signaling cascades relevant to the behavior of pancreatic cancer cells is accumulating at a rapid pace. Several examples are described below.

Raf/Mitogen-Activated Protein Kinase (MAPK) Cascade

Both activating K-ras mutations and growth factor receptor (eg, EGFR)-ligand interactions are relevant to activation of this cascade in pancreatic cancer. Activated Ras activates the Raf family of serine/threonine kinases, which in turn, through a series of phosphorylation events, activates MEK and its downstream effector extracellular signal-related kinase (ERK). ERK-mediated phosphorylation of its substrates promotes cell proliferation, survival, and differentiation.[19,27] Constitutive activation of this pathway may lead to increased growth, survival, and invasion of pancreatic cancer cells.[6] Although Ras itself is difficult to target, therapies directed at downstream effectors of this pathway deserve further investigation.[19]

Phosphoinositide 3-Kinase (PI3K)/AKT/Mammalian Target of Rapamycin (mTOR) Signaling Cascade

Phosphoinositide 3-kinase (PI3K) signaling can be activated by Ras as well as other growth factor-activated tyrosine kinase pathways.[19,28] Effectors of this pathway are activated and/or overexpressed in PDACs and mediate cell proliferation, survival, and chemoresistance signals.[6,19] Inhibitors of mammalian target of rapamycin (mTOR, eg, rapamycin) may be an effective strategy for targeting the downstream components of this pathway.[29,30]

Nuclear Factor Kappa B (NF-κB) Signaling Cascade

Nuclear factor kappa B (NF-κB) is a transcription factor that is constitutively active in nearly all pancreatic cancer cell lines and PDAC tissues.[7,19] NF-κB–regulated genes promote cell survival, invasion, chemoresistance, and angiogenesis.[6,19] Clinical trials are evaluating NF-κB inhibitors (eg, curcumin) in the treatment of pancreatic cancer.

Developmental Cascades

Hedgehog and Notch signaling cascades play critical roles in pancreatic organogenesis and development but are absent or display only very low levels of activity in the normal adult pancreas. Recent reports indicate that effectors of this pathway may play roles in initiation of pancreatic cancers and that they may represent therapeutic targets.[6,15, 31–34]

Telomerase

Telomerase is an enzyme that is implicated in the immortalization of human cancer cells; it is reported to be activated in 75% to 95% of PDACs.[16,35–40]

A progression model for pancreatic cancer

Evidence suggests that pancreatic cancer develops in a step-wise progression, in which a parallel series of histologic and genetic alterations occur that ultimately lead to invasive PDAC.[13,41–44] Based on studies of pathologic specimens, pre-invasive precursor lesions from which PDACs are hypothesized to arise have been termed pancreatic intraepithelial neoplasia (PanIN) lesions.[45] The now standardized pathologic classification system describes an increasing degree of cytologic and architectural atypia from PanIN-1 (lowest grade) to PanIN-3 (highest grade) lesions.[12,46] A PanIN-3 lesion is considered to be the equivalent of a “Tis” T-status (stage 0) lesion in the American Joint Committee on Cancer TNM System for Staging of Pancreatic Cancer. Telomere shortening has been detected in all grades of PanIN lesions and may be among the earliest genetic abnormalities to occur in the pathogenesis of PDAC.[47] Duct lesions with minimal cytologic and

architectural atypia also have been shown to have point mutations in the K-ras oncogene. K-ras mutation is therefore likely to be an early event in pancreatic carcinogenesis.[5,42] p16 inactivation is an intermediate event and inactivations of p53 and DPC4 appear to be late events in this progression model.[2,42,48,49]

High throughput profiling studies

The application of high-throughput methodologies is rapidly increasing the pace of discovery in this field.

For example, comparative genomic hybridization (CGH) has been used to identify genomic copy number alterations in pancreatic cancer.[50] Data from five studies that have identified chromosomal gains and losses using this technology are summarized in Table 2.[51–55] In another study, single nucleotide polymorphism (SNP) arrays allowed for detection of 41 homologous deletions (19 first reports) and 13 additional abnormal regions in PDAC cell lines.[56]

Numerous studies have applied various methods (eg, cDNA microarrays and genechips) for profiling transcript expression in PDACs.[57,58] Examples of genes found to be overexpressed in PDAC in multiple studies have been reviewed previously.[59] One such consistently overexpressed gene is carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6). CEACAM6 protein has been shown to be overexpressed in more than 90% of pancreatic adenocarcinomas; further, tumoral CEACAM6 expression status is negatively correlated with patient survival following surgical resection for PDAC.[60] In vitro and in vivo studies have demonstrated that CEACAM6 promotes cellular invasiveness, metastatic potential, and survival under anchorage independent conditions.[61,62] Furthermore, targeted therapy against CEACAM6 enhances chemosensitivity to gemcitabine and prolongs survival in a preclinical model of PDAC.[63–66]

Proteomic profiling studies of PDAC are also being reported. For example, using two-dimensional electrophoresis (2DE), Shen et al identified nine proteins that were unique to PDAC tissue specimens (annexin A4, cyclophilin A, cathepsin D, galectin-1, 14-3-3ζ, α-enolase, peroxiredoxin I, TM2, and S100A8).[67] Adding isotope-coded affinity tag (ICAT) to 2DE, Chen and colleagues were able to identify 151 proteins differentially expressed in pancreatic cancer.[68]

Models of pancreatic ductal cancer

Our understanding of the molecular biology of PDAC is derived from studies of pancreatic cancer cell lines, of human PDAC specimens, and of animal models of PDAC. Animal models include those generated through the administration of carcinogens (eg, injection of nitrosamines into Syrian golden hamsters) and implan-

Table 2 - Data from five studies that have identified chromosomal gains and losses using comparative genomic hybridization techniques.*

	Aguirre et al[52]	Bashyam et al[51]	Gysin et al [51]	Heidenblad et al[54]	Nowak et al[55]	
Recurrent chromosomal losses and deletions						
9p	X	X	X	X	X	**CDKN2A, TUSC3**
18q	X	X	X	X	X	**Smad4**
3p	X	X	X		X	**FHIT**, FOXP1
4q	X	X	X		X	**FBXW7**
6q	X	X	X		X	SEC63, **SASH1**, LPA
8p	X	X	X		X	DEFB103, DEFB105
17p	X	X	X		X	**TP53, MKK4**
6p		X	X		X	
13q	X		X		X	GPC5
21q	X		X		X	PARD6G
9q				X	X	**TNFSF15**
10q	X			X		CDH23
12q	X			X		**DUSP6**
15q			X		X	
21p			X		X	
22q	X				X	
Xp		X	X			
Recurrent chromosomal gains and amplifications						
12p	X	X	X	X		**KRAS2**
20q	X		X		X	CTSZ, NCOA3
8q	X		X	X		**MYC**
11q	X	X	X			**Cyclin D**
17q	X	X	X			**ERBB2**
7q		X		X	X	**SMURF1**
5p	X		X			BASP1
7p	X		X			**EGFR**
14q		X			X	**TGFB3**
6p		X		X		**NOTCH4**
19q		X		X		**AKT2, eIF3κ**
3q	X			X		

*Genes highlighted in boldface are known to be associated with pancreatic carcinoma.

tation of pancreatic cancer cells or tissue fragments into immunodeficient mice.
Recently, there has been significant progress in the development of transgenic (genetically engineered) animal models of pancreatic ductal carcinoma.[69,70] Several of these models include introduction of activating K-ras mutations into the pancreas. These K-ras mutations are sufficient to induce the development of pancreatic abnormalities similar to PanIN lesions. However, the lesions rarely develop into invasive adenocarcinomas.[71] In contrast, when activating K-ras mutations are introduced in the context of a second abnormality (eg, p16 or p53 mutation), mice develop PDAC and, in some cases, progression to metastatic disease.[12,70,72] A consensus report on genetically engineered mouse models of pancreatic exocrine neoplasias was recently published.[69]

Familial pancreatic cancer

As addressed previously in this review, pancreatic cancer, in general, is believed to develop as a result of a progressive series of sporadic mutations in the somatic genome. Less commonly, certain rare inherited conditions or germline mutations passed from parent to offspring can result in an elevated risk of pancreatic cancer.[9,73–75] Inherited mutation of the BRCA2 gene through the germline, for example, can significantly increase the risk of pancreatic cancer.[75,76]
Although sporadic mutation of the p16 gene is one of the signature genetic lesions of pancreatic cancer, interestingly, this mutation can also be inherited through the germline. The condition associated with this mutation inherited through the germline is known as familial atypical multiple-mole melanoma (FAMMM). Patients with this disorder develop nevi and melanomas, and have a 13- to 22-fold increased risk of developing pancreatic cancer during their lifetimes.[77] Several other rare inherited genetic disorders, including Peutz-Jeghers syndrome and a hereditary form of pancreatitis, increase the risk of developing pancreatic cancer. These and other familial conditions associated with pancreatic cancers are reviewed in detail in other excellent reviews.[9,73-75] A better understanding of these familial conditions may facilitate determination of the roles of specific gene mutations (such as p16) in the pathogenesis of pancreatic cancer.

Conclusions

Our knowledge of the molecular biology of pancreatic cancer is growing rapidly; the pace of discovery likely will continue to increase during the foreseeable future. The challenge will be to translate this growing body of information into clinically applicable strategies for early diagnosis and more effective therapies. It is possible to imagine a future in which PanINs are detected (using molecular biomarker-based imaging) in at-risk patients (identified through comprehensive profiling of germline mutations and analysis of environmental risk factors) and definitively

treated. Global profiling of the lesion would be used to individualize selection of a regimen of targeted therapies that would be used to halt disease progression. Although we remain optimistic, this scenario will become a reality only through a better understanding of the molecular biology of pancreatic cancer.

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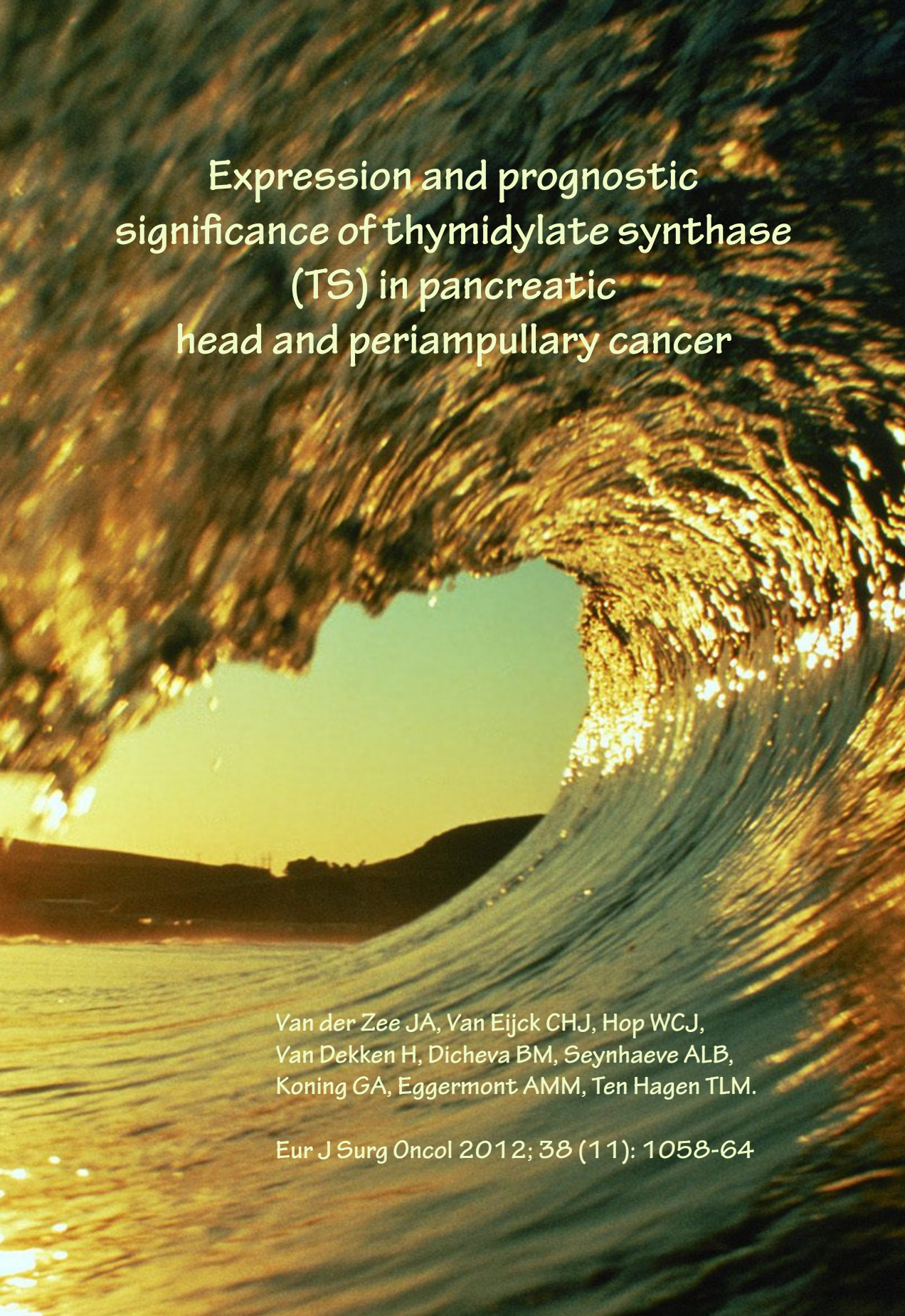

Expression and prognostic significance of thymidylate synthase (TS) in pancreatic head and periampullary cancer

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Abstract

Pancreatic cancer has a dismal prognosis. Attempts have been made to improve outcome by several 5-FU based adjuvant treatment regimens. However, the results are conflicting. There seems to be a continental divide with respect to the use of 5-FU based chemoradiotherapy (CRT). Furthermore, evidence has been presented showing a different response of pancreatic head and periampullary cancer to 5-FU based CRT. Expression of thymidylate synthase (TS) has been associated with improved outcome following 5-FU based adjuvant treatment in gastrointestinal cancer. This prompted us to determine the differential expression and prognostic value of TS in pancreatic head and periampullary cancer.

TS protein expression was studied by immunohistochemistry on original paraffin embedded tissue from 212 patients following microscopic radical resection (R0) of pancreatic head (n= 98) or periampullary cancer (n= 114). Expression was investigated for associations with recurrence free (RFS), cancer specific (CSS) and overall survival (OS), and conventional prognostic factors.

High cytosolic TS expression was present in 26% of pancreatic head tumours and 37% of periampullary tumours (p= .11). Furthermore, TS was an independent factor predicting favourable outcome following curative resection of pancreatic head cancer (p= .003, .001 and .001 for RFS, CSS and OS, respectively). In contrast, in periampullary cancer, TS was not associated with outcome (all p> .10).

In conclusion TS, was found to be poorly expressed in both pancreatic head and periampullary cancer and identified as an independent prognostic factor following curative resection of pancreatic head cancer.

Introduction

Pancreatic cancer is the fourth most common cause of cancer related deaths in the United States, accounting for approximately 37.000 deaths annually.[1] Radical surgery currently offers the only potential for cure. Because of its late presentation and its highly aggressive behaviour, only 20% of patients presenting with pancreatic cancer are amendable for resection, and even following radical resection both local and systemic recurrence remain a major problem.[2] Therefore adjuvant radiotherapy with or without chemotherapy has been studied to improve survival. In the 1960s, the Mayo Clinic already demonstrated that the addition of 5-fluorouracil (5-FU) to radiotherapy improves survival in locally unresectable gastrointestinal cancer.[3]

This initial study in the palliative setting led to several trials testing the value of 5-FU based chemoradiotherapy (CRT) in the adjuvant setting. The GITSG-trial was the first randomized trial to demonstrate a significant benefit of adjuvant 5-FU based CRT compared to surgery alone.[4] These results were supported by the single institution experience from Johns Hopkins Hospital and the recent collaborative study with Mayo Clinic.[5-7] In contrast, studies from Europe do not support the use of adjuvant 5-FU based CRT. In fact the ESPAC-1 even suggested a detrimental effect on survival compared to chemotherapy or surgery alone.[8,9] Interestingly, the EORTC trial performed a sub-analysis differentiating between pancreatic head- and periampullary cancer. It was in this subset-analysis that, in line with studies from the USA, a trend for improved survival following 5-FU based CRT was observed in patients with pancreatic head cancer. In contrast no such effect was observed for periampullary cancer.

5-FU was originally developed to target thymidylate synthase (TS), a rate limiting enzyme in the novel synthesis of DNA. Following its metabolization, 5-FU can bind TS thereby preventing binding of the normal substrate dUMP and inhibiting DNA synthesis.[10] The available TS pool could therefore be of interest in the response to chemotherapy.

Clinical evidence for a role of primary tumour TS levels in the response to adjuvant therapy was provided by studies in colorectal-,[11-15] gastric-,[16,17] and node positive breast cancer.[18] The value of TS in determining whether a patient will benefit from 5-FU based adjuvant therapy has also been investigated in pancreatic cancer, however the results are conflicting. Besides the predictive value, reports on the expression levels and prognostic value of TS are also inconsistent.[19-21]

The conflicting results with respect to the potential benefit of adjuvant 5-FU based CRT in pancreatic cancer, the difference between pancreatic head- and periampullary cancer and the inconsistent results with respect to the major target of 5-FU, TS, led to the design of the present study.

We aimed to determine the expression of TS in pancreatic head and periampullary cancer and evaluate its effect on outcome.

Patients and methods

Patient Population

Retrospectively, 231 patients treated for pancreatic adenocarcinoma with curative intent by Whipple's procedure or pylorus preserving pancreaticoduodenectomy at Erasmus Medical Center between 1987 and 2008, who had no microscopically residual tumour (R0), were identified. Tumours were classified by location having its origin either in the pancreatic head or periampullary region, the latter group comprising of tumours originating in the Ampulla of Vater or the distal common bile duct. Tumour samples originating before the new 2002 UICC TNM classification were reevaluated according to these new criteria.

Representative tumour areas were encircled on original hematoxylin/eosin slides by a GI pathologist with special expertise in pancreatic pathology and staining was performed on corresponding formalin fixed, paraffin embedded tissue.

During the above-mentioned period two randomized control studies were ongoing in our center. Between September 1987 and April 1995, 15 patients were randomized to the treatment arm of the EORTC 40891 trial, receiving two 2-weeks lasting courses of 5-FU as a continuous infusion (max 1500 mg/day, 5 days a week) together with radiotherapy (2 Gy/day, 5 days a week) with an interval of 2 weeks between the two cycles. From June 2000 up to its closure in March 2007, 28 patients were randomized to the treatment arm of a trial combining intra-arterial chemotherapy and radiotherapy. Patients received six 1-week lasting cycles of intra-arterial mitoxantrone (10 mg/m^2 on day 1), folinic acid (170 mg/m^2/day on days 2 and 4), 5-FU (600 mg/m^2/day on days 2 and 4) and cisplatinum (60 mg/m^2 on day 5), with an interval of 4 weeks between each cycle. In between the first and second cycle, patients received radiotherapy for 6 weeks (1.8 Gy/day, 5 days a week up to a total of 54 Gy). These trials and the results have been described in detail elsewhere.[8,22,23]

At the time of the present report, the median follow-up duration was 19 months (range 0-192 months). Recurrence free survival (RFS) was defined as the time from date of surgery to the date of first proof of disease recurrence (locally, distant or both) or to death without relapse. Overall survival (OS) was computed as the number of months from resection to death of any cause as registered by the social security death index (SSDI). For cancer specific survival (CSS) patients that died without recurrent disease were excluded. Patients who died in hospital following procedure related complications were excluded from analysis with respect to survival as their death was unrelated to tumour biology and would have introduced a confounding influence on survival analysis.

Thymidylate synthase (TS) expression by immunohistochemistry

Immunohistochemistry was performed according to the protocol used in clinical practice at our institution and was optimized for TS.

Briefly, 4 mM sections were deparaffinized in xylene and rehydrated through decreasing ethanol series ending in distilled water. Antigen retrieval was performed

by microwave heating (20 min preheating followed by 20 min of cooking) in Tris-EDTA buffer pH 9.0. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide (H_2O_2) in PBS for 20 min. Sections were incubated overnight at 4°C with a monoclonal mouse antibody to TS (TS106, Dako Netherlands B.V., Heverlee, Belgium) at 50x dilution in Dako REAL antibody diluent (S2022, Dako), which reduces background staining without any need for additional blocking steps. Following incubation with the secondary antibody (Dako REAL Envision HRP Rabbit/ Mouse) for 30 min at RT, immunostaining was developed by immersion in diaminobenzidine. Slides were washed extensively between each of the above-mentioned steps. Nuclei were counterstained with Harris Hematoxylin, followed by dehydration, fixation and finally covered using Leica multistainer and robotic coverslipper (ST5020 and CV 5030, Leica Microsystems B.V., Rijswijk, Netherlands). Positive and negative controls were included in each run.

Tissue evaluation

Slides were examined and scored separately by three observers (J.A.v.d.Z.; B.M.D. and T.L.M.t.H.) blinded to both clinical and pathological data. TS expression was quantified using a visual grading system based on the extent of staining. Immunoreactivity in the cytosol and nucleus were evaluated separately. Patients showed absent (<10% ductal cells positive), low (≥10% but <50% of positive cells) or high (≥50% positive cells) cytosolic TS expression in their tumours. Furthermore, TS was either absent (<10% nuclei from ductal cells positive) or present (≥10% positive nuclei) in the nuclei of tumour cells. Discrepant scores were resolved by consensus.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 for Windows.
Differences in distribution of categorical clinico-pathological parameters between groups were compared with Chi-square or Fisher's exact tests when appropriate. The distributions of RFS, CSS and OS were estimated using Kaplan Meier methodology. Analyses were stratified by location of the tumour. Univariate associations were tested using Log-rank test. Cox proportional hazards regression model was used to test whether the associations identified by univariate analysis were independent of other factors. The factors included in the multivariate analysis model beside the factor under investigation, TS, were tumour extension (T status), nodal involvement (N status), tumour differentiation and adjuvant treatment. The first three were included in the model because they are well established prognostic factors in pancreatic cancer; adjuvant treatment was included in the model to prove that any prognostic effect of TS identified, was not influenced by the adjuvant treatment given to some patients.
By the use of interaction terms it was investigated whether the prognostic effect of TS differed between pancreatic head and periampullary cancers.
All p values reported are two sided and values ≤ .05 were considered statistically significant.

Results

Patient population

Of 231 margin negative patients, 212 were adequately stained for analysis of thymidylate synthase expression. The age of the study population ranged from 36 to 87 years (median 65 years) and consisted of slightly more males (122/212) than females. Tumour origin was the pancreatic head in 98/212, the remainders were periampullary tumours. Nine patients died during postoperative stay and were therefore excluded from survival analysis.

Thymidylate synthase (TS) expression

TS was heterogeneously expressed between and within tumours. Cross-reactivity was observed with connective tissue elements (Fig. 1).

Thirty-seven percent of periampullary tumours showed high cytosolic TS expression compared with 26% of pancreatic head tumours (p= .11). Nuclear TS was observed in 43% of periampullary tumours and 21% of pancreatic head tumours (p= .001). In periampullary cancer, tumours with higher cytosolic TS expression were more likely to show nuclear expression (p< .001). In pancreatic head cancer, cytosolic and nuclear TS were not associated (p= .16).

Pathologic correlations

In adenocarcinoma of the pancreatic head, TS enzyme levels were not associated with conventional prognostic factors such as tumour extent (T status), nodal involvement and differentiation. In periampullary tumours however, an association of borderline significance was observed with tumour extent (p= .08) for cytosolic TS and a significant association with tumour extent for nuclear TS (p= .03).

Those tumours classified as T3 or T4 had lower TS levels than T1 and T2 tumours.

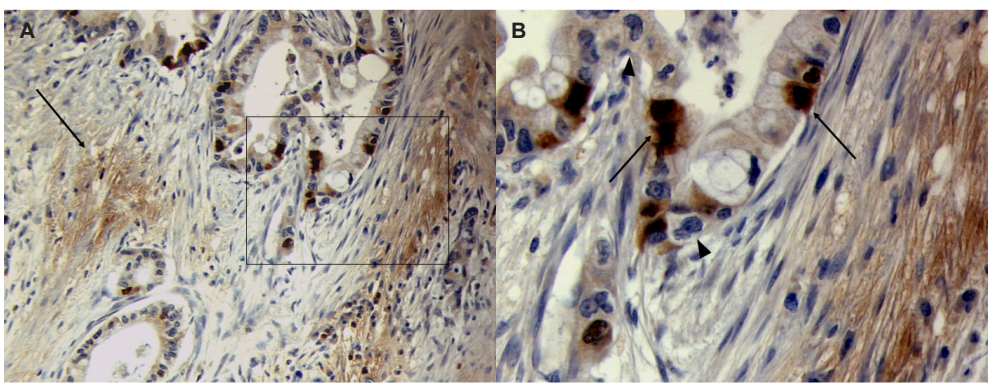

Fig. 1 – Pancreatic cancer tissue immunohistochemically stained with TS106 monoclonal antibody. A: The tumor shows heterogeneous expression of thymidylate synthase (TS) and minor cross reactivity with connective tissue (arrow). B: Magnification of area depicted in A clearly showing both positive- (arrow) and negative (arrowhead) nuclear and cytosolic staining (40x magnification).

Furthermore, adjuvant treatment was more often given to patients lacking or showing low TS than to patients with pancreatic head cancer showing high TS expression (p= .04). In the group of periampullary cancer, an equal amount of patients received adjuvant treatment.

Clinical correlations

Prognosis of patients treated for periampullary cancer was significantly better than prognosis of patients with adenocarcinoma of the pancreatic head (p< .001 for RFS, CSS and OS). 5-Year CSS of patients treated for periampullary cancer was 39% whereas CSS of patients treated for pancreatic head cancer was 16% (Fig. 2)

To test whether the association of TS expression with survival differed between pancreatic head and periampullary cancer, we tested for effect modification ("interaction") by tumour location in the Cox-models. This test showed a statistically different effect of cytosolic TS expression on survival between periampullary and pancreatic head tumours (p= .032, .031 and .019 for RFS, CSS and OS, respectively).

In patients treated for adenocarcinoma of the pancreatic head, higher cytosolic TS expression was correlated with improved RFS (p= .014), CSS (p= .020) and OS (p= .006) (Fig. 3) Cytosolic TS was not associated with outcome in periampullary cancer (p= .46, .56 and .60 for RFS, CSS and OS, respectively).

Multivariate analysis identified TS as an independent factor predicting improved outcome following radical resection (R0) of pancreatic head cancer (p= .003, .001 and .001 for RFS, CSS and OS, respectively). Conventional prognostic factors that predicted outcome in pancreatic head cancer were nodal involvement and tumour differentiation (Table 1). Both nodal involvement and tumour differentiation

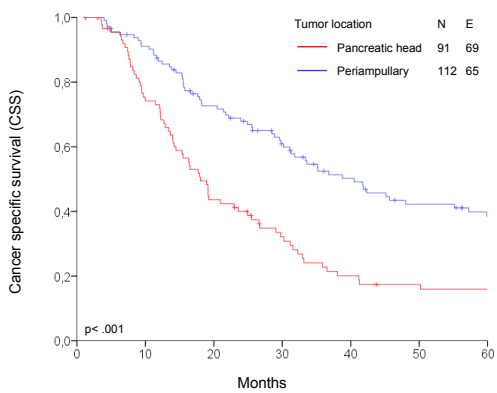

Fig. 2 – Cancer specific survival (CSS) of patients with adenocarcinoma of respectively the pancreatic head and periampullary region. Patients with pancreatic head cancer (—) were more likely to die of their disease than patients with periampullary cancer (—).

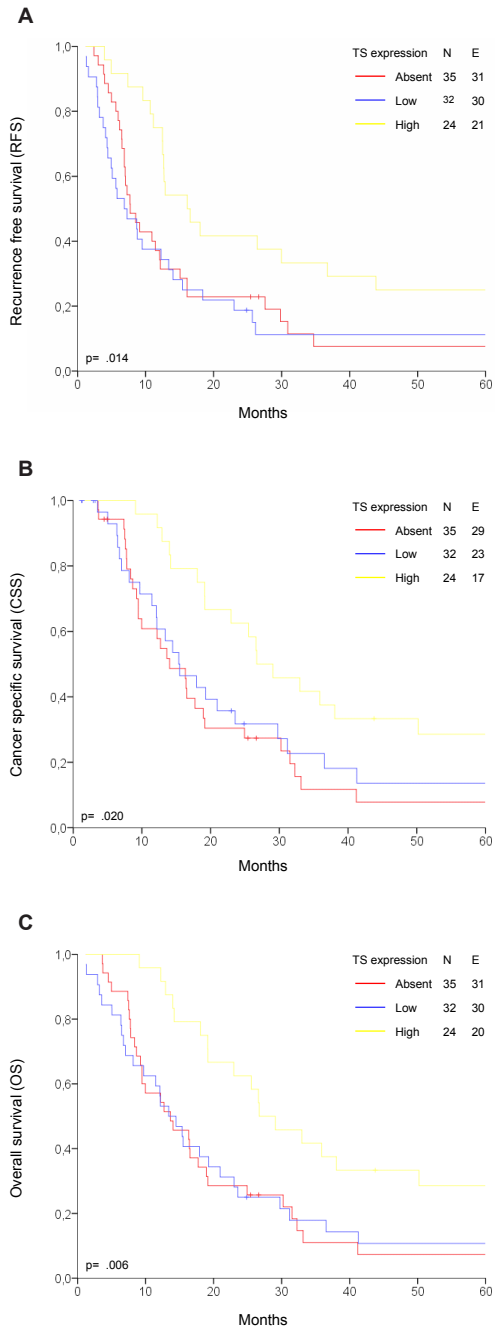

Fig. 3 – The effect of TS on prognosis of pancreatic head cancer. Patients with high tumor TS (—) had better A: recurrence free survival (RFS) B: cancer specific survival (CSS) and C: overall survival (OS), than patients lacking (—) or showing low TS (—) expression.

Table 1 – Multivariate analysis of conventional prognostic factors and TS in pancreatic head cancers.

Factor	N	RFS				CSS				OS			
		%5yr	HR	95% CI	p	%5yr	HR	95% CI	p	%5yr	HR	95% CI	p
Tumour extension					.069				.11				.14
T 1/2[a]	15	20				21				20			
T 3/4	74	12	1.80	0.96-3.37		15	1.76	0.80-3.52		13	1.63	0.86-3.09	
Nodal involvement					.019				.002				.002
No[a]	41	26				30				28			
Yes	50	3	1.82	1.10-3.00		3	2.29	1.35-3.87		2	2.22	1.35-3.65	
Differentiation					.016				.011				.029
Well[a]	35	8				36				36			
Moderately	32	11	2.00	0.97-4.11	.060	13	1.93	0.90-4.16	.09	11	2.02	0.98-4.17	.057
Poorly	24	25	3.44	1.48-8.03	.004	7	3.79	1.56-9.18	.003	7	3.09	1.36-7.60	.008
TS					.003				.001				.001
Absent[a]	35	8				8				7			
Low	32	11	1.02	0.60-1.73	.94	14	0.68	0.38-1.22	.19	11	0.85	0.50-1.45	.56
High	24	25	0.38	0.20-070	.002	29	0.29	0.14-0.56	.001	29	0.31	0.16-0.59	.001
Adjuvant therapy					.93				.76				.96
No[a]	65	17				19				17			
Yes	26	8	0.98	0.59-1.63		10	0.91	0.51-1.63		8	0.99	0.58-1.67	

[a] Reference category.

were also independent prognostic factors in periampullary cancer.
Nuclear TS was not associated with outcome in either tumour (data not shown).

Discussion

Thymidylate synthase (TS) tissue expression

This is the first study reporting the differential expression of thymidylate synthase (TS) in adenocarcinoma of the pancreatic head and periampullary region.
Expression of TS protein was determined by immunohistochemistry. Immunohistochemistry is a semiquantitative method, the advantage however, as compared to quantitative methods like Polymerase Chain Reaction (PCR) or western blot, is that one can identify the sub-cellular location of protein expression. In addition, one avoids contamination with non-malignant cells and the desmoplastic tissue, characteristic of pancreatic cancer.
High cytosolic TS expression (i.e. at least 50% of cells positive for TS) was present in 26% and 37% of pancreatic head and periampullary tumours, respectively.

These rates are lower than the 43, 47 and 63% reported in pancreatic cancer by Takamura, Formentini and Hu, respectively. In the study by Takamura and colleagues, high TS was defined as more than 25% of cells positive for TS, which could be a possible reason for the higher rate reported in their study. Overall, cut-off levels from 0% up to 75% have been reported.[12,13,21,24-39] Formentini and Hu used the area with the highest expression for classification of the tumour, increasing the probability of classifying a tumour as a high expressor. Furthermore, they graded expression by intensity, a method by which slight differences in experimental circumstances can have significant effects on outcome.
If, as suggested by several previous studies in colorectal-, [11-15] gastric-,[16,17] and breast cancer,[18] high TS levels would predict patients to derive benefit from 5-FU based adjuvant therapy, the relatively low percentage of patients actually expressing TS at a high level reported by us, suggests only few patients would benefit from the use of 5-FU in the adjuvant treatment of pancreatic cancer. However, the present study is a retrospective analysis including a sub-sample of two adjuvant therapy trials, that was neither prospectively designed nor powered to identify a difference in outcome with respect to adjuvant treatment. Furthermore, numbers were too small to perform exploratory analysis.

Prognostic value of thymidylate synthase (TS)
On initiation of this study, in addition to the expression of TS, another major interest of ours was to explore the effect of TS on prognosis in both adenocarcinoma of the pancreatic head and that of the periampullary region. Although the expression of TS was not significantly different between both tumour locations, we did observe a different effect on prognosis. Cytosolic TS proved to be an independent predictor of a favourable outcome following curative resection of pancreatic head cancer. Patients with pancreatic head cancer lacking or showing low levels of TS were respectively 3 and 2 times more likely to die from pancreatic cancer as compared to high expressing patients. In contrast, no association with outcome was observed for TS expression in the periampullary group. We have previously suggested that pancreatic head and periampullary tumours are distinct biological entities, based on a distinct prognostic effect of HMGA1 observed in both tumours.[40] The current TS data seem to support this hypothesis.
Our results, suggesting TS to positively affect prognosis in pancreatic head cancer, compare well to those obtained by Takemura and colleagues.[21] However, not all studies observed this effect.[19,20] In fact, the study by Hu et al. identified high TS as an independent predictor of poor prognosis. There is a possibility that this difference in effect on outcome is caused by the R1 cases included in their study, since Takemura and co-workers also observed a different effect between resectable and unresectable disease.[21]
The positive correlation of TS with outcome of pancreatic cancer identified by us and Takemura might be somewhat counterintuitive with respect to its role in the de novo synthesis of DNA and the opposite relation found by others in other cancers. However, in case of low TS expression levels, the salvage pathway might become the major source of DNA synthesis. Furthermore, TS functions as an RNA binding

protein, regulating expression of genes such as p53 and the myc family of transcription factors by translational repression.[41] It could be that in pancreatic cancer the end result of the interaction of TS with p53 and other transcription factors is inhibition of tumour growth. Finally, circulating or metastasized tumour cells with reduced TS might have adapted and overcome the low TS levels allowing them to survive, while cells with high TS levels are more stringent and may be less equipped to survive once they have metastasized.
In the present study, endpoints were also analyzed as a function of nuclear TS, however no association with outcome was observed. Relatively few studies report TS expression at the nuclear site, and none of them have performed an analysis on the relation with outcome.[34,42,43]

Conclusion
In summary, only a small proportion of pancreatic head and periampullary cancers show high TS expression. It was this group of patients that experienced a relatively favourable outcome following curative resection, compared with the ones lacking or showing low levels of TS. However, this was only true in patients diagnosed with pancreatic head and not periampullary cancer. This suggests that these tumours are different biological entities with respect to TS and might therefore also be differently affected by adjuvant treatment with 5-FU based CRT.

Conflict of interest statement
None declared.

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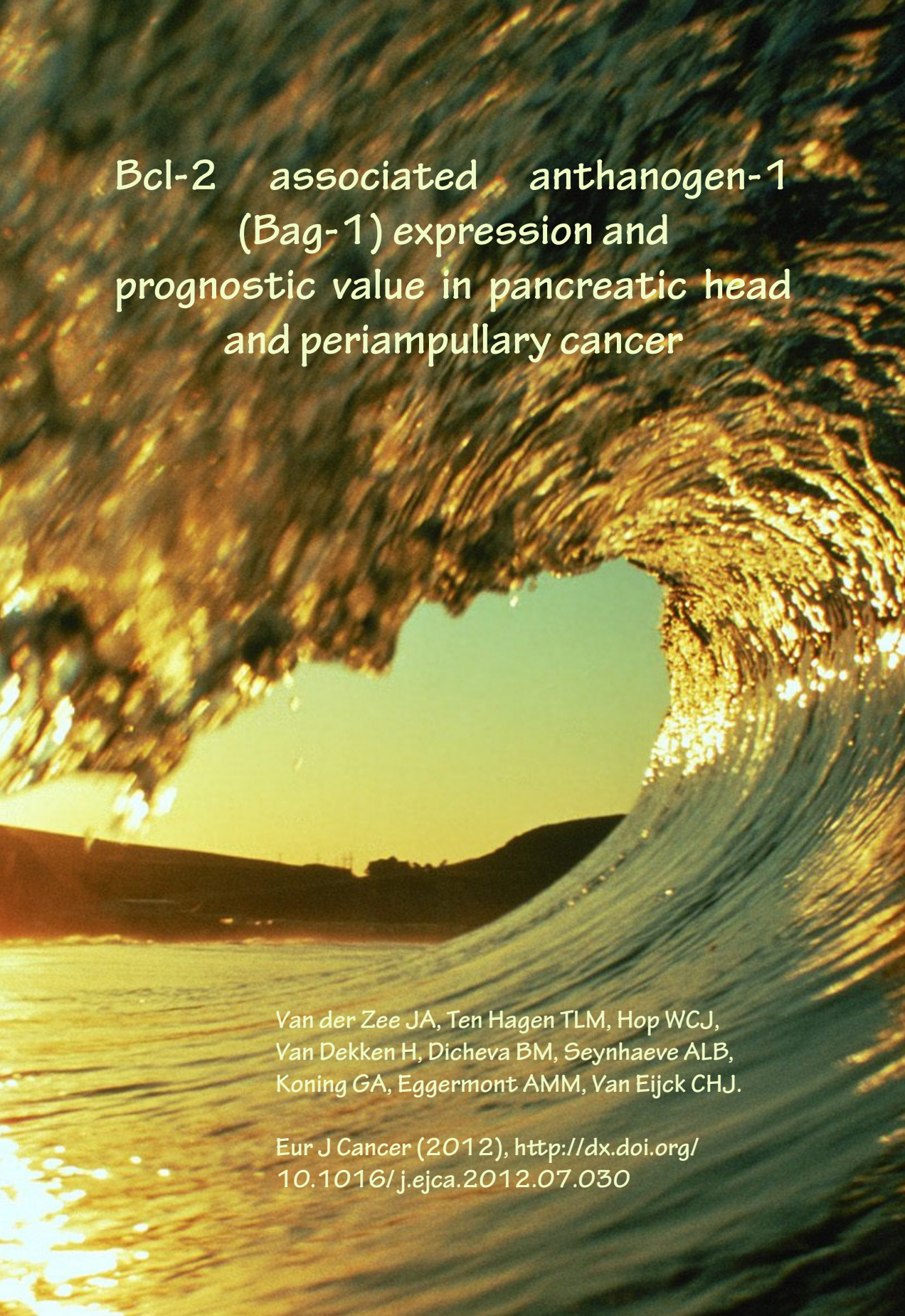

Bcl-2 associated anthanogen-1 (Bag-1) expression and prognostic value in pancreatic head and periampullary cancer

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Abstract

The expression of anti-apoptosis gene Bcl-2 associated anthanogen-1 (Bag-1), has been associated with outcome in several cancer types, however its prognostic role in pancreatic cancer is unknown. Aim was therefore to evaluate expression of Bag-1 in two anatomically closely related however prognostically different tumours, pancreatic head- and periampullary cancer and correlate expression with outcome.

Bag-1 protein expression was studied by immunohistochemistry on original paraffin embedded tissue from 217 patients with microscopic radical resection (R0) of adenocarcinoma of the pancreatic head or periampullary region. Expression was assessed for associations with recurrence free- (RFS), cancer specific- (CSS), overall survival (OS) and conventional prognostic factors.

Nuclear Bag-1 was present in 80% of tumours. In 40% Bag-1 resided in the cytosol, which was almost exclusively associated with nuclear expression. Nuclear Bag-1 protein was identified as an independent factor predicting a favourable outcome following radical resection of pancreatic head cancer. Eighteen percent of patients with nuclear Bag-1 were recurrence free and alive 5 years following surgery compared to none of the patients lacking expression. In periampullary cancer Bag-1 was not associated with outcome.

In conclusion, Bag-1 was present in the majority of both pancreatic head- and periampullary cancers. However it was only identified as a discriminator of outcome in pancreatic head cancer.

Introduction

With an annual death rate of 34.000 and a 5-year survival of 5%, pancreatic cancer is the 4th leading cause of cancer-related death.[1] In spite of extended resections and several adjuvant chemo- and/or radiotherapy regimens, almost half of the patients develop recurrent disease within the 1st year.[2] Delineation of cancer phenotypes based on molecular markers of therapeutic responsiveness and overall outcome can enable stratification of patients to appropriate individualised therapeutic regimens, so that optimal treatment is given without delay and unnecessary adverse side-effects are minimised.[3]
One such candidate marker could be Bcl-2 associated anthanogen-1 (Bag-1) that was identified in the search for novel interaction partners of Bcl-2.[4] Besides apoptosis, Bag-1 is involved in cell signalling, stress-response/ protein degradation, proliferation and transcription.[5] Characterisation of RNA and protein showed that Bag-1 is generated as four isoforms and is overexpressed in multiple cancer cell lines.[6] Furthermore, Bag-1 has been associated with outcome in several cancer types.[7–17]
Our aims were therefore to determine the expression of Bag-1 in two anatomically closely related, however prognostically different tumours, pancreatic head and periampullary cancer and to elucidate its effect on outcome.

Patients and methods

Patient population
Two hundred and thirty-one patients treated for pancreatic adenocarcinoma with curative intend at Erasmus Medical Center between January 1987 and 2008 who had no microscopically residual tumour (R0, i.e. ≥1 mm tumour free margin) were retrospectively identified. Tumours were classified by location having its origin either in the pancreatic head or periampullary region, the latter group comprising of tumours originating in the Ampulla of Vater or the distal common bile duct. Tumour samples originating before the 2002 UICC TNM classification were re-evaluated according to these new criteria. Representative tumour areas were encircled on original haematoxylin/eosin slides by a GI pathologist specialised in pancreatic diseases and staining was performed on corresponding formalin fixed, paraffin embedded tissue.
During the above-mentioned period two randomised control studies were ongoing in our centre. Between September 1987 and April 1995, 17 patients were randomised to the treatment arm of the European Organisation for Research and Treatment of Cancer (EORTC) 40891 trial. From June 2000 up to its closure in March 2007, 31 patients were randomised to the treatment arm of a trial combining intra-arterial chemotherapy and radiotherapy. Both trials and the results have been described in detail elsewhere.[18,19]

At the time of the present report, the median follow-up duration was 19 months (range 0–192 months). Recurrence free survival (RFS) was defined as the time from date of surgery to the date of first proof of disease recurrence (locally, distant or both) or to death without relapse. Overall survival (OS) was computed as the number of months from resection to death of any cause as registered by the social security death index (SSDI). For cancer specific survival (CSS) patients that died without recurrent disease were excluded. Patients who died in hospital following procedure related complications were excluded from analysis with respect to survival as their death was unrelated to tumour biology and would have introduced a confounding influence on survival analysis.

Bag-1 expression by immunohistochemistry

Immunohistochemistry was performed as previously described by us,[20] with the use of a monoclonal mouse antibody to Bag-1 (5C5, ab49454, Abcam, Cambridge, United Kingdom (UK)) at 1500x dilution. Positive and negative controls were included in each run. The antibody was tested on Western Blot with commercially available Bag-1 overexpressing cell lysate (H00000573-T01, Abnova Corporation Taipel, Taiwan).

Tissue evaluation

Tumour areas were examined and scored independently by three investigators (J.A.v.d.Z., T.L.M.t.H. and B.M.D.) blinded to both clinical and pathological data. Bag-1 immunoreactivity in the nucleus and the cytosol were evaluated separately and scored as previously described with a slight modification to nuclear scoring with the addition of an extra group because of the high expression observed on slide screening.[13] Patients showed absent (≤10% nuclei of ductal cells positive), low (between 10 and 50% positive nuclei) or high (>50% nuclei positive) nuclear Bag-1 expression in their tumours. Furthermore, Bag-1 was either absent (≤10% ductal cells positive) or present (>10% positive) in the cytosol of tumours. In cases of disagreement consensus was reached by joint review.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 for Windows (IBM). Differences in distribution of categorical clinico-pathological parameters between groups were compared with Chi square or Fisher's exact tests when appropriate. The distributions of RFS, CSS and OS were estimated using Kaplan Meier methodology. Because of a significantly different effect of Bag-1 on outcome of pancreatic head- and periampullary cancer by interaction terms in the Cox models (p= .013, .038 and .022 for respectively RFS, CSS and OS), survival analyses were stratified by location of the tumour. Univariate associations were tested using Log-rank test. Cox proportional hazards regression model was used to test whether the associations identified by univariate analysis were independent of other factors. The factors included in the multivariate analysis model besides the factor under investigation, Bag-1, were tumour extent (T status), nodal involvement (N status) and tumour differentiation.

All p values reported are two sided and values ≤ .05 were considered statistically significant.

Results

Patient population

Of 231 R0 patients, 217 patients had tissue slides that were suitable for analysis. The median age at time of surgery was 65 years (range 36–84 years) and there were slightly more male patients than females (126 versus 91). Forty-seven percent of patients were diagnosed with adenocarcinoma of the pancreatic head and in 53% the origin was periampullar. Ten patients died during postoperative stay and were thus excluded from survival analysis.

Bag-1 expression

Bag-1 was heterogeneously expressed between and also within tumour samples (Fig. 1). In adenocarcinoma of both the pancreatic head and periampullary region Bag-1 isoforms resided predominantly in the nucleus. Nuclear immunostaining was present in 80/102 (78%) pancreatic head tumours and 91/115 (79%) periampullary tumours, whereas only 35% (36/102) of the pancreatic head and 44% (50/115) of the periampullary tumours showed cytosolic Bag-1 expression. Bag-1 rarely resided solely in the cytosol, only five tumours showed cytosolic expression without nuclear expression.

Pathologic correlations

Nuclear Bag-1 was not associated with any of the conventional prognostic factors

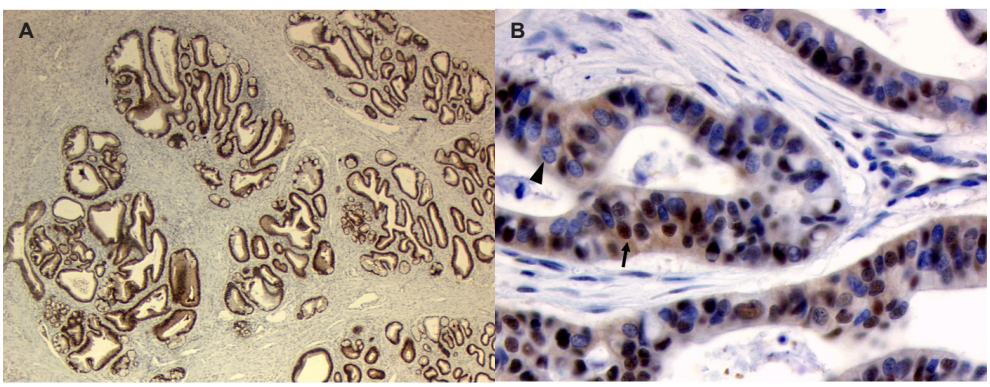

Fig. 1 – Pancreatic cancer tissue immunohistochemically stained with Bcl-2 associated anthanogen-1 (Bag-1) antibody. (A) Overview of tumour area (2.5x). (B) Magnification of a tumour heterogeneously expressing Bag-1 as shown by both positive- (arrow) and negative (arrowhead) nuclear and cytosolic staining (40x).

in either tumour. In contrast cytosolic Bag-1 was associated with smaller periampullary tumours (p= .014) and a trend towards less nodal involvement than periampullary tumours lacking cytosolic Bag-1 expression (p= .084). Cytosolic Bag-1 was not associated with T-, N- stage or tumour differentiation in pancreatic head cancer.

Clinical correlations

In pancreatic head cancer, nuclear Bag-1 was significantly associated with better outcome measures (p= .003, .004 and .004 for respectively RFS, CSS and OS). Twenty percent and 17% of patients with respectively high and low nuclear Bag-1 were recurrence free and alive 5 years following complete resection of the tumour, whereas all of the patients lacking expression had died (Fig. 2). Following correction for conventional prognostic factors such as tumour extent (T status), nodal involvement and degree of differentiation, nuclear Bag-1 proved to be an independent predictor of RFS and OS (p= .023 and .041, respectively) and showed a trend for an association with CSS (p= .058) (Table 1). In contrast, no relation with outcome was observed for periampullary tumours (p= .55, .64 and .48 for respectively RFS, CSS and OS).

Positive cytosolic Bag-1 showed a trend for a favourable outcome following radical

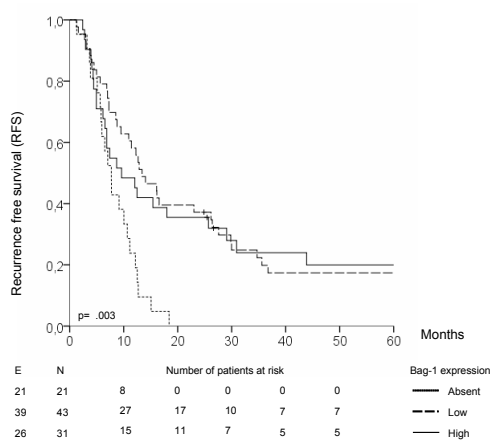

Fig. 2 – Kaplan Meier curves of recurrence free survival (RFS) of patients treated for pancreatic head cancer. Survival was better in patients with tumours showing high (——) or low (– – –) nuclear Bcl-2 associated anthanogen-1 (Bag-1) than patients with cancers lacking (- - - - -) expression (p= .003).

resection of pancreatic head cancer, however only for two out of three outcome measures (p= .07, .13, .07 for respectively RFS, CSS and OS). For periampullary cancer no such trend was observed (p= .13, .17 and .16 for respectively RFS, CSS and OS).

Table 1 – Multivariate analysis of conventional prognostic factors and Bcl-2 associated anthanogen (Bag-1) on outcome in pancreatic head cancers.

Factor	N	Recurrence free survival (RFS)				Cancer specific survival (CSS)				Overall survival (OS)			
		%5yr	HR	95% CI	p	%5yr	HR	95% CI	p	%5yr	HR	95% CI	p
Tumour extension					.45				.70				.65
T 1/2[a]	15	20				21				20			
T 3/4	78	13	1.27	0.68-2.25		16	1.14	0.58-2.26		14	1.15	0.62-2.15	
Nodal involvement					.13				.050				.057
No[a]	45	26				31				28			
Yes	50	3	1.45	0.89-2.37		3	1.68	1.00-2.83		3	1.61	0.99-2.62	
Differentiation					.17				.14				.15
Well[a]	15	33				38				33			
Moderately	62	10	1.82	0.94-3.49	.07	13	1.83	0.88-3.79	.11	11	1.86	0.97-3.55	.062
Poorly	17	12	1.90	0.88-4.10	.10	12	2.35	1.00-5.52	.050	12	1.91	0.88-4.12	.10
Bag-1					.023				.058				.041
Absent[a]	21	0				0				0			
Present	74	18	0.53	0.30-0.92		22	0.57	0.31-1.02		19	0.56	0.33-0.98	

%5yr, % 5 year survival by univariate analysis; HR, hazard ratio; 95% CI, 95% confidence interval.
[a] Reference category.

Discussion

This is the first study assessing the expression and clinical significance of Bag-1 in pancreatic head and periampullary cancer.

Approximately 80% of pancreatic head and periampullary cancers showed positive Bag-1 protein expression. In the majority of tumours, Bag-1 resided in the nucleus. In fact, cytosolic localisation was almost exclusively associated with nuclear expression. Expression patterns did not differ between tumours of the pancreatic head and periampullary region. The observed preference of Bag-1 for the nucleus is in line with several studies in breast-, colorectal- and prostate cancer.[7,8,15,17] However, there are also reports of Bag-1 residing predominantly in the cytosol.[10–13]

The data presented, demonstrate that nuclear Bag-1 protein is an independent factor predicting a favourable outcome following radical resection of pancreatic head cancer. Eighteen percent of patients with nuclear Bag-1 were recurrence free and alive 5 years following surgery whereas all patients lacking expression had died. The positive prognostic value of Bag-1 is in line with a previous study in lung cancer,[12] however in that study it was cytosolic rather than nuclear Bag-1 predicting outcome. In the current study only a trend was observed for an association of cytosolic Bag-1 with outcome following resection of pancreatic head cancer.

The prognostic impact of Bag-1 has been investigated predominantly in breast cancer. Although the first study reported poor outcome for patients with tumours expressing Bag-1 in the nucleus,[10] following studies indicated a favourable out-

come for patients expressing Bag-1, which is in agreement with our observations.[11,16,17]

Differently, in colorectal cancer Bag-1 expression was associated with poorly differentiated tumours, advanced stage, metastasis and poor prognosis.[7,8,13] Likewise in tongue cancer Bag-1 was associated with advanced stage and worse outcome.[9] Studies investigating Bag-1 expression in prostate cancer observed high nuclear Bag-1 in hormone refractory patients and cytosolic Bag-1 was associated with shorter time to disease progression.[14,15]

Bag-1 is a multifunctional protein that has four isoforms, all translated from the same mRNA, initiated at different sites. All isoforms have a subcellular location of preference, however they can translocate from one compartment to the other depending on the protein it associates with. In addition to Bcl-2, Bag-1 has been shown to associate with Hsp70, nuclear hormone receptors, RAF-1 kinase, components of the ubiquitylation/proteosome machinery and DNA.[5,6] Bag-1 isoforms and its subcellular location and thus its interaction partners are tissue specific, which might explain the variable effect of Bag-1 protein on outcome in different tumours.

It might be somewhat counterintuitive that an anti apoptotic protein is associated with improved outcome, however not surprising, since for Bcl-2, its first identified interaction partner, the same phenomenon was observed.[21] Furthermore, Hsp-70 has been shown to compete with Raf-1 for binding to Bag-1. Under stressful circumstances Bag-1/Raf-1 complexes are replaced by Bag-1/Hsp70 complexes, resulting in inhibition of DNA synthesis and cell growth.[22]

The differential effect of Bag-1 on prognosis of pancreatic head and periampullary cancer, previously also observed for high mobility group A1 (HMGA1),[20] suggests that although anatomically closely related with stem cells resembling each other, pancreatic head- and periampullary cancer are two different entities with different molecular signatures and behaviour. In case of Bag-1 the different behaviour is probably caused by the specific isoforms present, its subcellular location and consequently the molecules it interacts with. Isoform specific antibodies are therefore needed to provide further insight into the possible pathways involved in both types of cancer.

In conclusion, nuclear Bag-1 is expressed in a significant proportion of pancreatic head and periampullary cancers and could differentiate between patients with pancreatic head cancer that might benefit and those that are not likely to benefit from radical resection. In fact, patients lacking expression had such poor survival that resection should be questioned. These patients at least seem to require a more radical approach. However, unfortunately alternatives are currently limited. Further exploration of the multiple isoforms of Bag-1 is needed in order to understand what makes Bag-1 a predictor of favourable outcome in pancreatic head cancer.

Conflict of interest statement

None declared.

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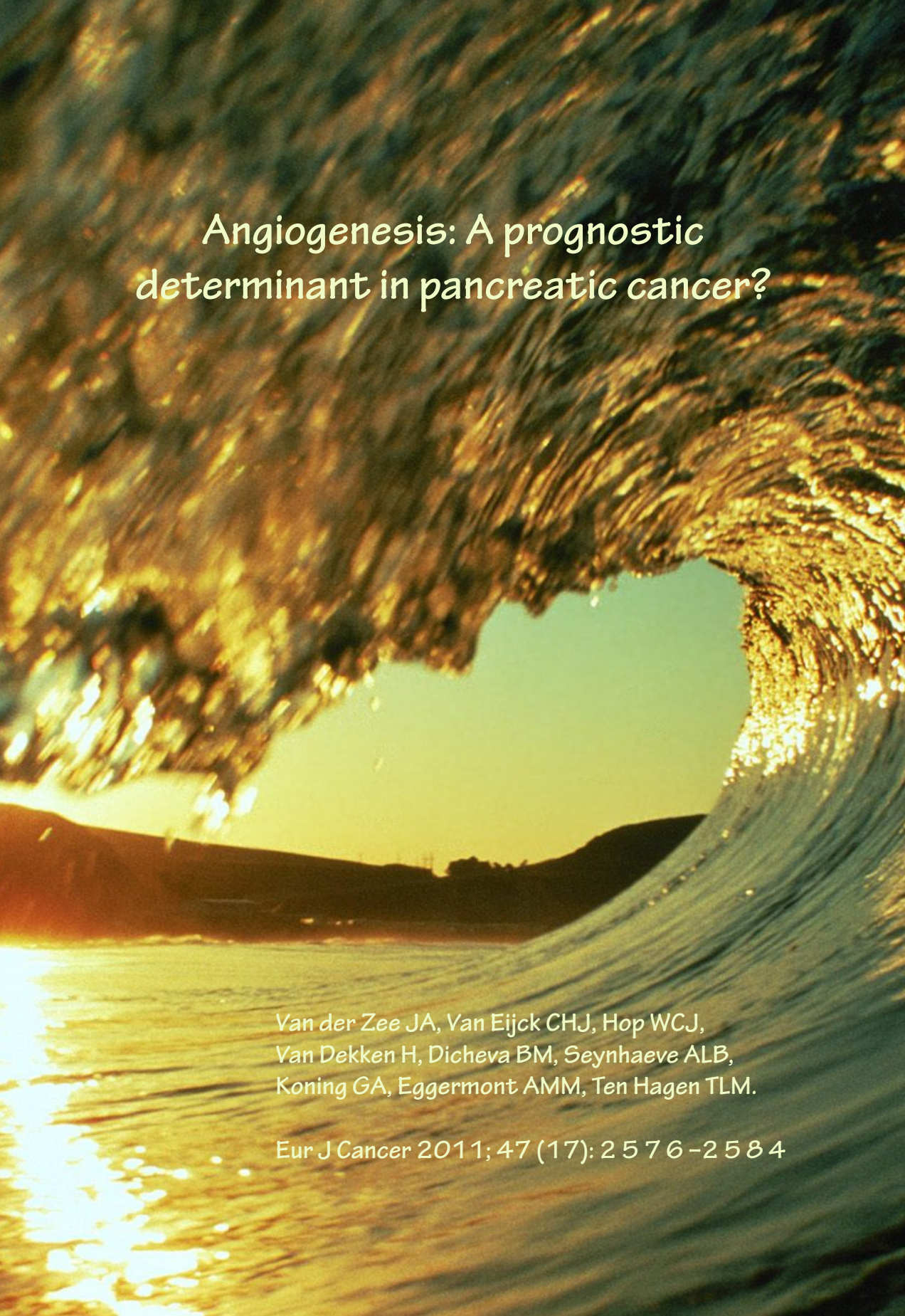

Angiogenesis: A prognostic determinant in pancreatic cancer?

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Eur J Cancer 2011; 47 (17): 2576-2584

Abstract

Angiogenesis has been associated with disease progression in many solid tumours, however the statement that tumours need angiogenesis to grow, invade and metastasise seems no longer applicable to all tumours or to all tumour subtypes. Prognostic studies in pancreatic cancer are conflicting. In fact, pancreatic cancer has been suggested an example of a tumour in which angiogenesis is less essential for tumour progression.

The aim of the present study was therefore to measure angiogenesis in two anatomically closely related, however prognostically different types of pancreatic cancer, pancreatic head and periampullary cancer, and investigate its relation with outcome.

Vessels were stained by CD31 on original paraffin embedded tissue from 206 patients with microscopic radical resection (R0) of pancreatic head (n= 98) or periampullary cancer (n= 108). Angiogenesis was quantified by microvessel density (MVD) and measured by computerised image analysis of three randomly selected fields and investigated for associations with recurrence free survival (RFS), cancer specific survival (CSS), overall survival (OS) and conventional prognostic factors.

MVD was heterogeneous both between and within tumours. A higher MVD was observed in periampullary cancers compared with pancreatic head cancers ($p < .01$). Furthermore, MVD was associated with lymph node involvement in pancreatic head ($p = .014$), but not in periampullary cancer ($p = .55$). Interestingly, MVD was not associated with RFS, CSS or with OS.

In conclusion, angiogenesis is higher in periampullary cancer and although associated with nodal involvement in pancreatic head cancer, pancreatic cancer prognosis seems indeed angiogenesis independent.

Introduction

Pancreatic cancer has a highly invasive and metastatic potential. Up to 80% of patients present with locally advanced disease or distant metastasis, precluding them from resection. And even following curative resection, recurrent disease remains a major problem, almost half of the patients relapse within the first year.[1] Several attempts have been made to improve outcome by adjuvant treatment regimens, however with disappointing results.[2]

Disease progression through to the formation of metastasis is a highly selective multi-step process, dependant on both tumour characteristics and environmental factors. Angiogenesis plays an important role in tumour growth and progression by supplying necessary oxygen, growth factors and nutrients, as well as by facilitating the dissemination of tumour cells.[3–7] In an attempt to translate observations from experimental models to clinical practice, quantification of tumour vessels has been performed and correlations with clinicopathological factors and outcome have been investigated. Irrespective of the method used, the amount of tumour microvessels was associated with recurrent disease and poor survival in several primary tumours, including melanoma,[8] breast,[9–19] lung cancer,[20–22] prostate,[23] bladder,[24] ovary,[25] colorectal carcinoma, [26] hepatocellular carcinoma[27] and also in their metastatic counterparts.[28] However, not all studies identified a negative relation with outcome,[29–37] a study on colorectal cancer[38] and one on node negative breast cancer[39] even suggested a beneficial effect of a higher microvessel density on prognosis. Quantification of tumour microvessels has also been performed in some relatively small sized studies on pancreatic cancer, however again results are conflicting.[40–48]

Not all tumours need angiogenesis to grow, invade and metastasise. Some tumours apply alternative mechanisms such as co-option, mosaicism, vasculogenesis or intussusceptive vascular growth, to obtain blood vessels.[49] Furthermore, the role of angiogenesis as a prognostic marker cannot be generalised to all tumour types. Pancreatic cancer was opted an example of a tumour type that is less dependent on angiogenesis for tumour progression. Even within a certain type of cancer, subtypes are not necessarily equally dependant on angiogenesis for their growth and progression. Gastric cancer is an example in which the process of growth and metastasis of one subtype, diffuse type gastric cancer, is less angiogenesis dependent than the other, intestinal type gastric cancer.[50,51]

The aim of the present study was therefore to quantify tumour angiogenesis in a large cohort of two anatomically closely related however prognostically distinct types of pancreatic cancer, pancreatic head and periampullary cancer and elucidate whether angiogenesis is a prognostic marker in either of these types of pancreatic cancer.

Patients and methods

Patient Population

Retrospectively 231 patients treated for pancreatic adenocarcinoma with curative intent at Erasmus Medical Center between 1987 and 2008, who had no microscopically residual tumour (R0), were identified. Tumours were classified by location, having its origin either in the pancreatic head or periampullary region, the latter group comprising of tumours originating in the Ampulla of Vater or the distal common bile duct. Tumour samples originating before the new 2002 UICC TNM classification were re-evaluated according to these new criteria.

Representative tumour areas were encircled on original haematoxylin/eosin slides by a GI pathologist with special expertise in pancreatic pathology and staining was performed on corresponding formalin fixed, paraffin embedded tissue.

During the above-mentioned period two randomised control studies were ongoing in our center. Between September 1987 and April 1995, 17 patients were randomised to the treatment arm of the European Organisation for Research and Treatment of Cancer (EORTC) 40891 trial, receiving two courses of 5-FU as a continuous infusion (max 1500 mg/day) followed by radiotherapy (20 Gy). From June 2000 up to its closure in March 2007, 32 patients were randomised to the treatment arm of a trial combining intra-arterial chemotherapy and radiotherapy. Patients received six cycles of intra-arterial mitoxantrone (10 mg/m^2), folinic acid (170 mg/m^2/day), 5-FU (600 mg/m^2/day) and cisplatinum (60 mg/m^2), the first cycle followed by radiotherapy (54 Gy). These trials and the results have been described in detail elsewhere.[52–54]

At the time of the present report, the median follow-up duration was 19 months (range 0–192 months). Recurrence free survival (RFS) was defined as the time from date of surgery to the date of first proof of disease recurrence (locally, distant or both) or to death without relapse. Overall survival (OS) was computed as the number of months from resection to death of any cause as registered by the social security death index (SSDI). For cancer specific survival (CSS) patients that died without recurrent disease were excluded. Patients who died in hospital following procedure related complications were excluded from analysis with respect to survival as their death was unrelated to tumour biology and would have introduced a confounding influence on survival analysis.

CD31 expression by immunohistochemistry

Immunohistochemistry was performed according to the protocol used in clinical practice at our institution and was optimised for CD31.

Briefly, 4 µM sections were deparaffinised in xylene and rehydrated through decreasing ethanol series ending in distilled water. Antigen retrieval was performed by microwave heating (20 min preheating followed by 20 min of cooking) in Tris–ethylene diamine tetra-acetic acid (EDTA) buffer pH 9.0. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide (H_2O_2) in PBS for 20 min. Sections were incubated overnight at 4°C with a ready to use mouse monoclonal antibody to CD31 (JC70A, Dako Netherlands B.V., Heverlee, Belgium). Followed

by incubation with the secondary antibody (Dako REAL Envision HRP Rabbit/Mouse) for 30 min at room temperature, immunostaining was developed by immersion in diaminobenzidine. Slides were washed extensively between each of the above steps. Nuclei were counterstained with Harris Haematoxylin, followed by dehydration, fixation and finally covered using Leica multistainer and robotic coverslipper (ST5020 and CV 5030, Leica Microsystems B.V., Rijswijk, Netherlands). Positive and negative controls were included in each run.

Microvessel density (MVD) analysis

Tumour areas were examined with a Leica DM-RXA microscope. Three random x160 fields (x16 objective, x10 ocular) were captured using a Sony 3CCD DXC 950 camera connected to a computer. Images were analysed using UTHSCSH Image Tool. Briefly, colours were separated and the 24bits binary blue image was transferred to grayscale followed by setting an automatic threshold that clearly identified the CD31 positive endothelial cells. Fixed light intensity was used throughout the analysis. MVD was recorded from each of the three high-power fields and either the highest count or the average was used for analysis. MVD was calculated as the number of CD31 positive pixels per picture.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 for Windows. Differences in MVD between groups were compared with Student's t-test or ANOVA when appropriate. The correlation between MVD and established prognostic factors was carried out using linear regression analysis. In these analyses of MVD, logarithmically transformed values (base 10) were used for an approximate normal distribution.

The distributions of RFS, CSS and OS were estimated using Kaplan–Meier curves. In one model the median MVD was used as potential prognostic cut-off; in another the 25th, 50th and 75th percentile were evaluated. Univariate associations were tested using Log-rank test. Cox proportional hazards regression model was used to test whether outcome measures were independent of other established prognostic factors, such as tumour extension (T-status), mode of differentiation and nodal status. All p values reported are two sided and values ≤ .05 were considered statistically significant.

Results

Patient population

Tissue blocks were available from 222 out of 231 patients. Sixteen slides could not be scored due to poor quality. As a result immunostaining was correlated with established prognostic factors for 206 cases. Ten patients died during postoperative stay and were thus excluded from survival analysis. The age of the patients ranged from 36 to 87 years (median 65 years) and the study population included

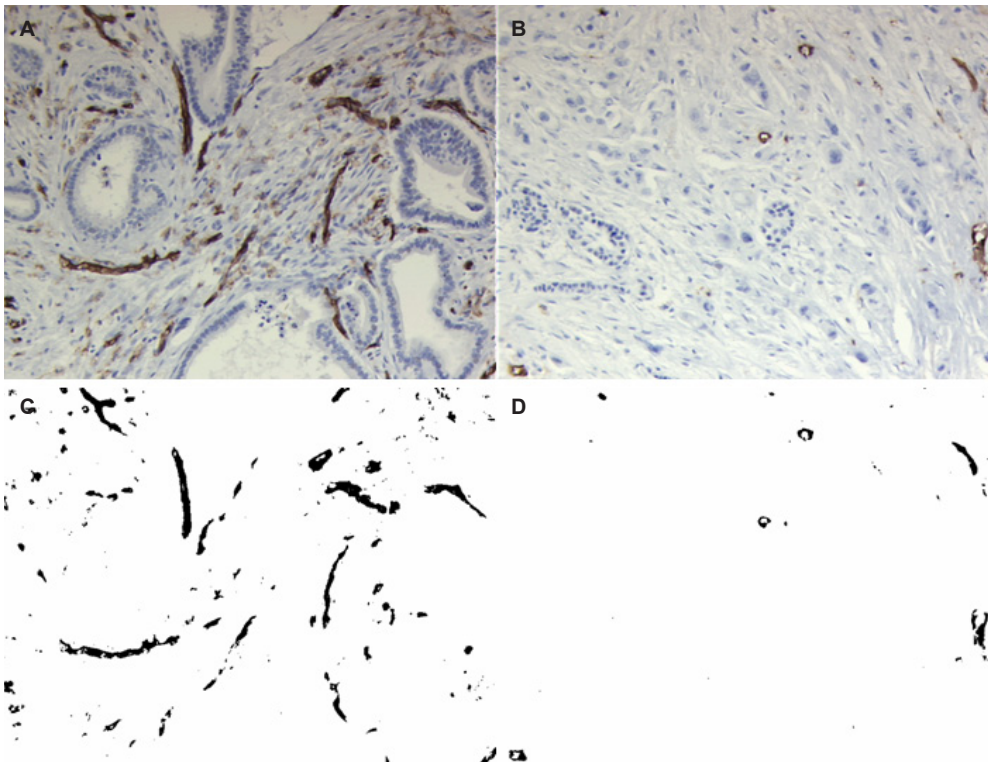

Fig. 1 – Two examples of pancreatic cancer tissue immunohistochemically stained with CD31 antibody (A and B) (16x magnification) and their binary overlays (C and D) showing a tumour with respectively high (A) and low microvessel density (MVD) (B).

slightly more males than females (113 versus 83). Ninety-one tumours originated in the head of the pancreas whereas 105 patients had periampullary cancer.

Microvessel density (MVD)

ANOVA showed large variations between tumours (SD= 0.24) and between the different random spots within a tumour (SD= 0.22). Therefore the average values per tumour were calculated and used for further analysis. The average MVD from the three pictures analysed, ranged from 0.3 to 12.4 percent/patient (mean 3.1; median 2.7).We also calculated the highest MVD measured among the three spots. The highest MVD ranged from 0.5 to 13.6 percent/patient (mean 4.4; median 3.8).

Both the average and the highest MVD were not normally distributed. In Fig. 1, two examples are given of such a random field. The first represents a patient with an average MVD of 3.6, the second a patient with an average MVD of 0.9. Fig. 2 represents the vessel distribution in two spots of normal pancreas demonstrating rather homogeneous vessel distribution.

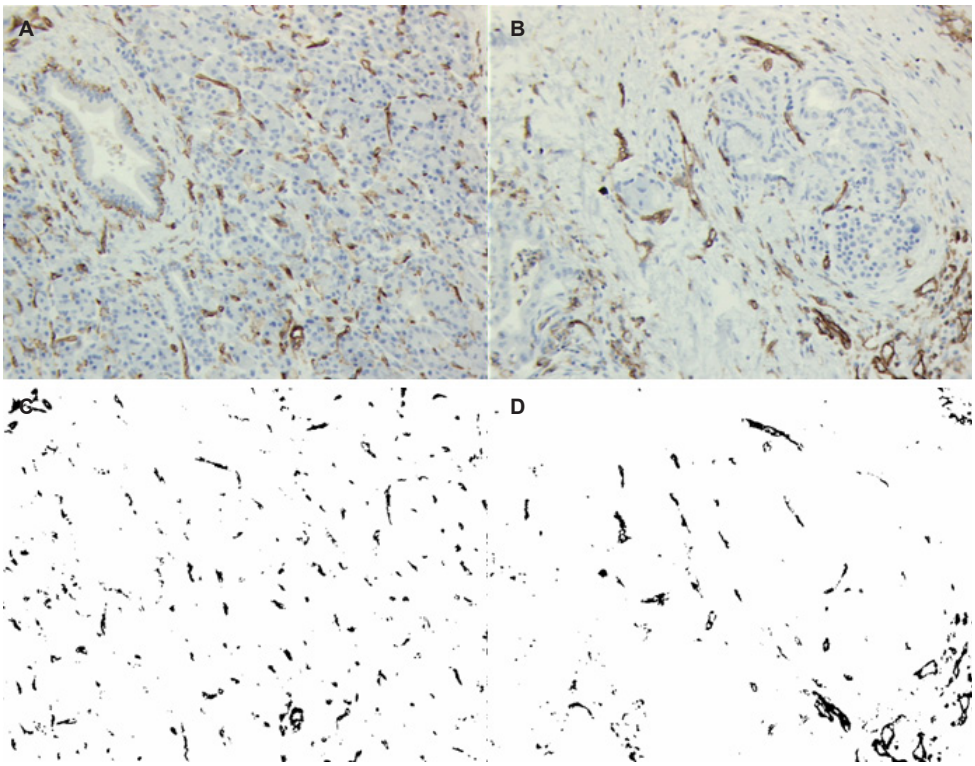

Fig. 2 – Two examples of normal pancreas tissue immunohistochemically stained with CD31 antibody (A and B) and their binary overlays (C and D) (16x magnification) suggesting homogeneous vessel distribution.

Pathologic correlations

The average MVD of pancreatic head tumours was less than MVD of tumours originating in the periampullary region (respectively 2.4 and 2.9 percent; p< .01). Furthermore, nodal status correlated with the average MVD in pancreatic head tumours (p= .014), whereas this was not the case in perampullary tumours (p= .55). In pancreatic head tumours with lymph node involvement a larger proportion of the tumour was occupied by vessels (Fig 3). There was no correlation with T-status or with mode of differentiation. This was true for both pancreatic head and periampullary cancer.

When the highest instead of the average MVD was used for analysis, findings were similar to analysis using the average MVD. However, in addition to nodal status, mode of differentiation also correlated with vessel area (p= .047). Generally, moderately and poorly differentiated pancreatic head cancers were more vascularised than well-differentiated pancreatic head cancers.

Clinical correlations

All conventional prognostic factors proved highly significant in predicting RFS,

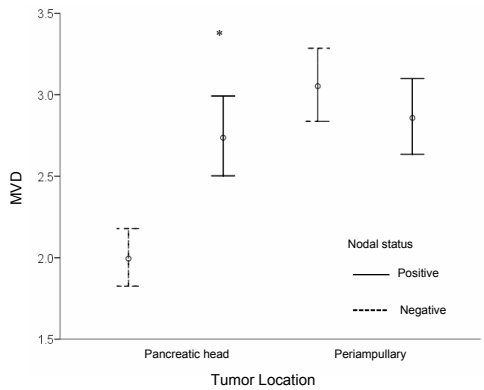

Fig. 3 – Microvessel density (MVD) (±1 SE) in node positive (——) and node negative (- - - -) tumours for respectively pancreatic head and periampullary cancer. In patients with nodal involvement tumours showed a higher proportion of vessels than those with clean lymph nodes. However this was only true for pancreatic head cancer (*p= .014).

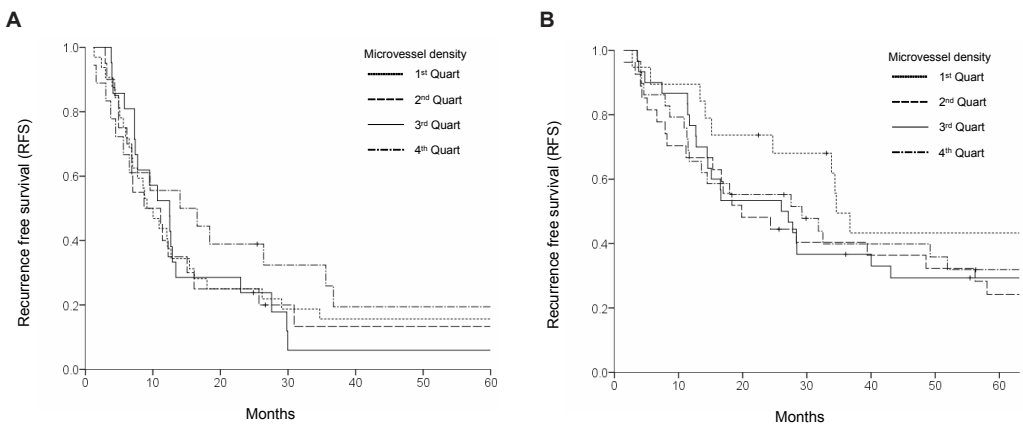

Fig. 4 – Recurrence free survival (RFS) of patients treated for adenocarcinoma of respectively the pancreatic head (A) and periampullary (B) region. Microvessel density (MVD) did not have an impact on RFS of pancreatic cancer (p= .77).

CSS and OS in univariate analysis. In multivariate analysis however, tumour extension (T status) lost its prognostic value for all outcome measures. The type of pancreatic cancer, i.e. pancreatic head or periampullary cancer, proved to be the strongest independent predictor of outcome (data not shown).

To test whether the relation between MVD and the outcome measures differed between the two types of pancreatic cancer, we tested for effect modification ('interaction') by tumour type in the Cox-models. This test showed that the type of

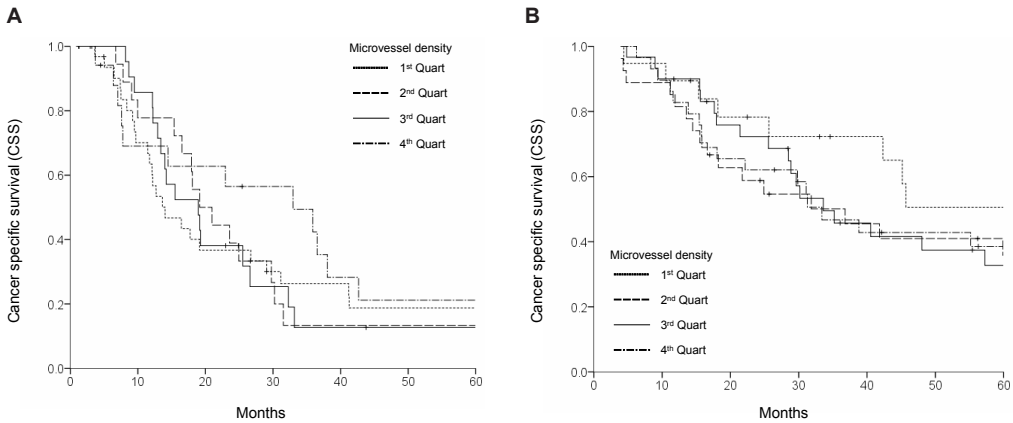

Fig. 5 – Cancer specific survival (CSS) of patients treated for adenocarcinoma of respectively the pancreatic head (A) and periampullary (B) region. Microvessel density (MVD) did not have an impact on CSS of pancreatic cancer (p= .79).

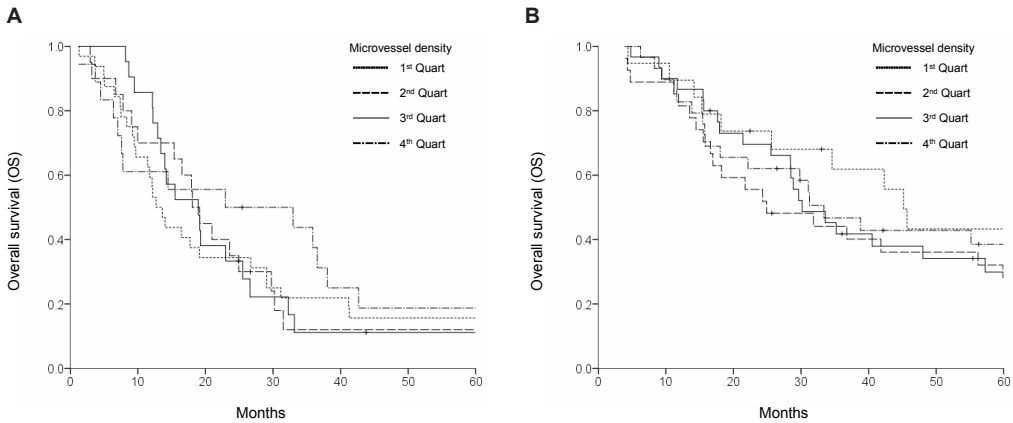

Fig. 6 – Overall survival (OS) of patients treated for adenocarcinoma of respectively the pancreatic head (A) and periampullary (B) region. Microvessel density (MVD) did not have an impact on OS of pancreatic cancer (p= .84).

pancreatic cancer did not determine the effect of MVD on outcome (all p> .19 for RFS, CSS and OS). The same was done for the highest MVD measured, with similar conclusions. Therefore, for survival analysis, both tumours were treated as one group, however for direct interpretation purposes Kaplan–Meier curves are presented for the two tumour types separately.

MVD was divided into quartiles and the four groups were tested for trend regarding relation with outcomes. No trend for an association of MVD with RFS (p= .77),

CSS (p= .79) or OS (p= .84) was observed (Figs. 4–6). In fact none of the four groups showed a significant difference with any of the other groups following pair wise comparison. Following multivariate analysis, only tumour type, nodal status and differentiation proved to be independent prognostic factors for RFS, CSS and OS. MVD provided no additional prognostic information (Table 1). Furthermore, the effect of MVD on outcome did not significantly depend on N-category either (all p> .21 for RFS, CSS and OS).
All analyses were repeated with the highest MVD instead of the average MVD with similar conclusions (data not shown).

Discussion

This is the largest study to date, quantifying tumour angiogenesis and evaluating its effect on the prognosis of pancreatic cancer.
The pan-endothelial marker CD31 was used to stain tumour vessels and the proportion of vessel to tumour area (MVD) was measured using an automated system to quantify tumour angiogenesis. MVD appeared to be heterogeneous between, but also within tumours, while vessel density in normal pancreatic tissue appeared more homogeneous.

Table 1 – Multivariate analysis of conventional prognostic factors and microvessel density (MVD) on outcome in pancreatic cancer.

Factor	N	Recurrence free survival (RFS)				Cancer specific survival (CSS)				Overall survival (OS)			
		%5yr	HR	95% CI	p	%5yr	HR	95% CI	p	%5yr	HR	95% CI	p
Tumour type					.001				.001				.001
Pancreatic head[a]	91	14				17				15			
Periampullary	105	31	0.49	0.35-0.68		38	0.49	0.34-0.71		34	0.49	0.35-0.68	
Tumour extension					.09				.13				.08
T 1/2[a]	50	44				48				45			
T 3/4	142	15	1.43	0.95-2.15		21	1.44	0.90-2.29		18	1.45	0.96-2.20	
Nodal involvement					.001				.001				.001
No[a]	92	37				42				28			
Yes	104	10	1.89	1.35-2.67		16	2.07	1.41-3.03		2	1.77	1.26-2.50	
Differentiation					.003				.002				.008
Well[a]	32	43				51				33			
Moderately	129	21	1.49	0.94-2.37	.09	26	1.66	0.98-2.84	.06	11	1.61	1.01-2.57	.047
Poorly	34	12	2.55	1.47-4.41	.001	16	2.89	1.57-5.33	.001	12	2.42	1.39-4.22	.002
MVD			1.00[b]	0.83-1.19	.96		0.93[b]	0.77-1.13	.48		0.95[b]	0.79-1.13	.54

HR: Hazard ratio; %5 year: % 5 year survival by univariate analysis; 95% CI: 95% confidence interval
[a] Reference category
[b] Effect of doubling MVD

Furthermore, a higher MVD was observed in periampullary cancer as compared to pancreatic head cancer. Microvessel recruitment is the result of the angiogenic potential of the tumour cell itself and its interaction with the surrounding extracellular matrix. The angiogenic potential of a tumour cell is likely a reflection of its origin. Although both pancreatic head and periampullary cancer have a close anatomic relation, they have different stem cell origins. This difference in origin could explain the different molecular characteristics and behaviour of both tumours, reflected by the currently observed difference in MVD but also the previously observed differences in expression rates of c-erb,[55] p53, Ki-67,[56] SMAD 4[57] and HMGA1 and the effect on prognosis of both tumours.[58]

No association of MVD with other prognostic factors could be identified in periampullary cancer, whereas in pancreatic head cancer, a higher MVD coincided with lymph node involvement. Interestingly, Khan and co-workers observed the exact opposite: an association with nodal involvement in ampullary cancer instead of pancreatic cancer.[45] When the highest MVD measured was used for analysis, MVD was associated with both nodal status and differentiation. This is in line with another study in pancreatic cancer in which the vascular surface density and the number of vessels per mm^2 stroma correlated with poor histological differentiation.[44] The same investigators also found a relationship between MVD and tumour size and in yet another study an association with stage was observed.[41] In other tumours associations with tumour size,[11,12,14,18,27,35,36] T status,[17] differentiation[9,11–13,17,23,35,36] or lymph node status[9,11,12,18,36] were observed in some studies but not in others.[15,24–26,31–34,38]

Following correlation with established prognostic factors, the association of MVD with survival was assessed. Although we intended to evaluate tumour angiogenesis as a prognostic factor in pancreatic head and periampullary cancer separately, no interaction was identified between the type of pancreatic cancer and outcome. A separate analysis was therefore not justified. Consequently we analysed both types as one group. A possible reason for the lack of an interaction between tumour type and outcome could be the small difference between the actual MVD, 2.4 and 2.9 percent for pancreatic head and periampullary cancer, respectively, classifying both as relative hypoxic tumours.

In our cohort of patients with pancreatic cancer, MVD did not provide additional prognostic information to conventional prognostic factors such as nodal involvement and degree of differentiation. This is in line with some reports on the prognostic role of MVD in pancreatic cancer,[40,41,46] however in sharp contrast to others.[41–45,47,48]

Possible reasons for these inconsistent results are not limited to the difference in study population, but may be due to poor standardisation of the methodology. The assessment of MVD is generally a tedious process with poor reproducibility. First, as originally proposed by Weidner and colleagues, the most vascularised area (the so called 'hot-spot') needs to be selected. Then, each separate microvessel needs to be counted. Both selection processes are highly subjective and consequently likely associated with inter-observer variation. Furthermore, exclusively counting vessels neglects the vessel size aspect, and might underestimate the

metastatic potential of a tumour as hypothesised by Nagakawa et al. who showed that liver metastasis occurred more often when tumour invasion was present in the middle sized and the large vessels compared to the smaller sized ones.[46] An observation already made by Liotta and co-workers in the early seventies. They found a linear relationship between the number of vessels >30 μm and the number of tumour cell- and cell clump washout following perfusion of a xenografted fibrosarcoma.[3] However, the disadvantage of using vessel size to quantify MVD is again the subjective bias and interobserver variability associated with counting individual vessels.

To reduce the subjective bias and interobserver variability associated with selecting a hot-spot and counting individual microvessels, in the current study areas were selected randomly and analysed by automated computerised image analysis. Conveniently, the pixel/area aspect of the analysis performed does not completely neglect the vessel size aspect discussed above since a large vessel contains more pixels than a small vessel. Finally, the average of the randomly selected areas was taken to correct for the heterogeneous nature of tumours. Another reason for the differences observed between studies could be the antibody or agent used to detect the vessels. Antigen specificity is a major problem when staining endothelial cells. In case of CD34, lymphatic vessels, perivascular stromal cells as well as other stromal elements can be stained. The disadvantage of factor VIII is that it is absent on part of the capillary endothelium in tumour tissue. Disadvantages associated with staining for CD31, are the co-staining of inflammatory cells and frequent antigen loss due to fixatives that contain acetic acid. However inflammatory cells are easy to distinguish from endothelial cells and microwave antigen retrieval effectively abolishes the problem of antigen loss.[59] Furthermore, JC-70, the antibody used in the current study, has the advantage over factor VIII of being present also on immature blood vessels. Consequently, counts using this marker are 30% higher than those using factor VIII.[16] In 1996 an international consensus was developed by the experts in the field of MVD assessment. In this consensus CD31, the agent used in the current study, was chosen as the agent of choice for assessment of MVD on paraffin sections.

The lack of an association with prognosis could be explained in several ways. First, pancreatic cancer is known to be a hypoxic tumour.[60–63] Pancreatic cancer is characterised by an extensive extracellular matrix deposition, called desmoplasia, creating increased intratumoural pressure and tumour-vessel distances, resulting in a hypoxic environment.[64] Quite some research has been done in the last decennium on the role of pancreatic stellate cells in this extracellular matrix deposition. Recently one of the expert groups suggested that this hypoxic and fibrotic environment of pancreatic cancer might be due to both fibrogenic effects of pancreatic stellate cells and anti-angiogenic effects of cancer cells.[65] Evidence has been presented that hypoxia might act as a physiological selective agent against apoptosis- competent cells in tumours, thus promoting the clonal expansion of cells that acquire mutations in their apoptotic programs.[66]

Secondly, angiogenesis is but one step in the multi-step process of tumour progression and metastasis formation. It might be necessary but not sufficient to

produce metastasis.
In conclusion, the present study shows that periampullary cancer is more vascularised than pancreatic head cancer and although angiogenesis as quantified by microvessel density correlates with nodal involvement in pancreatic head cancer and thus gives some information on the malignant potential, pancreatic cancer seems indeed an example of a tumour in which prognosis is not dependent on angiogenesis.
This would also explain the poor response to anti-VEGF treatment observed in pancreatic cancer.[67,68]

Conflict of interest statement

None declared.

Acknowledgements

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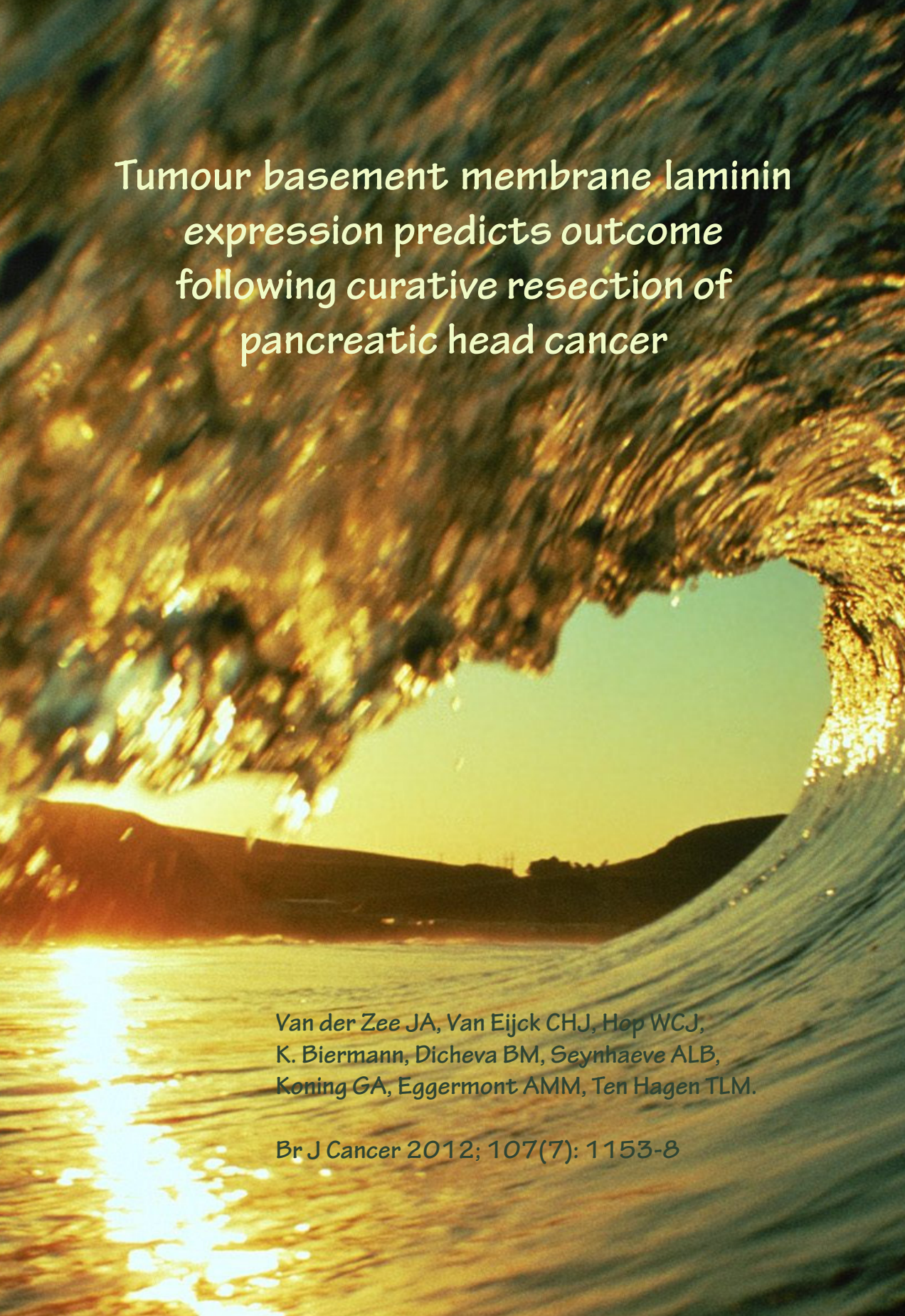

Tumour basement membrane laminin expression predicts outcome following curative resection of pancreatic head cancer

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Abstract

Although widely fragmented BMs have been associated with adverse outcome in several cancer types, comparatively little is known with respect to its effect on the prognosis of pancreatic cancer. The aim of the current study was therefore to determine the prognostic value of tumour basement membrane (BM) continuity in two anatomically closely related, however, prognostically different tumours, pancreatic head- and periampullary cancer.

Tumour BM continuity was determined by immunohistochemical staining of its two major components, laminin and collagen type IV. Associations were made with recurrence free survival (RFS), cancer-specific survival (CSS), overall survival (OS) and conventional prognostic factors.

Fifty-nine and 61% of pancreatic head and periampullary tumours, respectively, showed limited BM laminin expression. Whereas 43% and 41% of pancreatic head and periampullary cancers, respectively, showed limited BM collagen type IV expression. Limited BM laminin was associated with poor outcome following curative resection of pancreatic head cancer ($p= .034$, .013 and .017 for RFS, CSS and OS, respectively). Two and a half times as many patients with ≥ 25% BM laminin were recurrence free and alive 5 years following resection compared with those with limited BM laminin. Although staining patterns of both BM components were weakly correlated with each other, BM collagen type IV expression was not significantly associated with outcome in either tumour type.

In conclusion discontinuous BMs, determined by laminin expression, are associated with poor outcome following curative resection of pancreatic head cancer.

Introduction

Pancreatic cancer is one of the most lethal human cancers. Resection currently offers the only potential for cure. Owing to locally advanced disease or the presence of distant metastasis, only 20% of patients are amendable for resection and even following resection, recurrence remains a major problem (Li et al, 2004). In fact, almost half of the patients develop recurrent disease within the first year (Boeck et al, 2007).
Cancer progression and the formation of metastasis is a complex multi-step process coordinated by the dynamic interaction of tumour cells with their environment. Under normal circumstances epithelial cells are separated from the surrounding stroma by a highly crosslinked and insoluble sheet-like structure called the basement membrane (BM). Its two major components are laminin and collagen type IV, where laminin is the centre piece of the network and collagen type IV provides the scaffold. Apart from its barrier function, BMs provide structural support and regulate cell behaviour. Basement membranes are dynamic rather than static strutures, being continuously remodelled by glycoprotein rupture and synthesis. Tumour BMs are significantly less crosslinked and therefore more susceptible to proteolysis, remodelling and turnover than BMs of normal tissue (Liotta et al, 1983; Martinez- Hernandez and Amenta, 1983; Kalluri, 2003).
Conceivably, BM continuity is the net effect of tumour matrix interaction and consequently a reflection of tumour behaviour. Widely fragmented BMs have been associated with poor outcome in bladder- (Conn et al, 1987; Daher et al, 1987; Schapers et al, 1990), colorectal- (Forster et al, 1984; Forster et al, 1986; Havenith et al, 1988), lung (ten Velde et al, 1991) and hepatocellular cancer (Grigioni et al, 1991). An irregular and discontinuous deposition of type IV collagen and laminin has also been observed for BMs of pancreatic cancer (Mollenhauer et al, 1987; Lee et al, 1994; Imamura et al, 1995; Shimoyama et al, 1995; Linder et al, 2001), however, except for the study by Linder et al, expression patterns were not studied for their association with outcome.
We therefore decided to study the distribution of BM laminin and collagen type IV in relation to conventional prognostic factors and clinical behaviour of two anatomically closely related, however, prognostically different pancreatic tumours, pancreatic head and periampullary cancer.

Patients and methods

Patient Population
Retrospectively, 231 patients treated for pancreatic ductal adenocarcinoma with curative intend at Erasmus Medical Center between 1987 and 2008, who had no microscopically residual tumour (R0), were identified. Tumours were classified by location, having their origin either in the pancreatic head or periampullary region,

the latter group comprising of tumours originating in the Ampulla of Vater or the distal common bile duct. Tumour samples originating before the new 2002 UICC TNM classification were re-evaluated according to these new criteria.
Representative tumour areas were encircled on original haematoxylin/eosin slides by a GI pathologist (KB) with special expertise in pancreatic pathology and staining was performed on corresponding formalin-fixed, paraffin-embedded tissue.
During the above-mentioned period two randomised control trials were ongoing in our centre. Between September 1987 and April 1995, 17 patients were randomised to the treatment arm of the EORTC 40891 trial, receiving two courses of 5-FU as a continuous infusion (max 1500 mg per day) followed by radiotherapy (20 Gy). From June 2000 up to its closure in March 2007, 32 patients were randomised to the treatment arm of a trial combining intra-arterial chemotherapy and radiotherapy. Patients received six cycles of intra-arterial mitoxantrone (10 mg/m^2), folinic acid (170 mg/m^2 per day), 5-FU (600 mg/m^2 per day) and cisplatinum (60 mg/m^2), the first cycle followed by radiotherapy (54 Gy). These trials and the results have been described in detail elsewhere (Smeenk et al, 2007; Morak et al, 2008).
At the time of the present report, the median follow-up duration was 19 months (range 0–192 months). Recurrence-free survival (RFS) was defined as the time from date of surgery to the date of first proof of disease recurrence (locally, distant or both) or to death without relapse. Overall survival (OS) was computed as the number of months from resection to death of any cause as registered by the social security death index, whereas for cancer specific survival (CSS) only the pancreatic cancer-related deaths were counted. Patients who died in hospital following procedure related complications were excluded from analysis with respect to survival, as their death was considered unrelated to tumour biology. This was verified by an evaluation of in hospital death in relation to the tumour variable BM.

Expression of BM components by immunohistochemistry
Immunohistochemistry was performed according to the protocol used in clinical practice at our institution and was optimised for laminin and type IV collagen.
Briefly, 4 µM sections were deparaffinised in xylene and rehydrated through decreasing ethanol series ending in distilled water. In case of collagen type IV staining, antigen retrieval was performed by microwave heating (20 min preheating followed by 20 min of cooking) in Tris-EDTA buffer pH 9.0, whereas for laminin staining, proteinase K was applied for 10 min. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in PBS for 20 min. Sections were incubated overnight at 4 °C with a monoclonal mouse antibody to collagen type IV (CIV 22, M0785, Dako Netherlands BV, Heverlee, Belgium) or laminin (4C7, reacts with laminin alpha5; M0638, Dako) at dilutions of 40x and 20x respectively, in Dako REAL antibody diluent (S2022, Dako), which reduces background staining without the need for additional blocking steps. Following incubation with the secondary antibody (Dako REAL Envision HRP Rabbit/Mouse) for 30 min at room temperature, immunostaining was developed by immersion in diaminobenzidine. Slides were washed extensively between each of the above steps. Nuclei were

counterstained with Harris Haematoxylin. Next, slides were dehydrated, fixated and finally covered using Leica multistainer and robotic cover slipper (ST5020 and CV 5030, Leica Microsystems BV, Rijswijk, The Netherlands). Positive and negative controls were included in each run.

Tissue evaluation

Slides were examined by light microscopy and scored separately by three observers (JAvdZ; BMD and TLMtH) blinded to both clinical and pathological data. As previously described by Havenith et al (1988) and ten Velde et al (1991), the expression of BM components was quantified using a visual grading system based on the percentage of epithelial cell lining. The epithelial cell lining was either <25% (i.e., limited), 25–75% or >75%.

With respect to laminin expression inter-observer agreement was moderate (the weighted kappa ranged from 0.48 to 0.56), whereas inter-observer agreement for collagen type IV was less good (weighted kappa ranged from 0.34 to 0.48). Discrepant scores were resolved by consensus.

Basement membranes can only properly be identified by electron microscopy; with their 40–60nm thickness, they are beyond the resolving power of the light microscope. Although BM zone might therefore have been more appropriate to describe the observed epithelial cell lining by its major components laminin and collagen type IV (Kefalides et al, 1979); because of the wide use of the term BM in other studies, this was also used throughout the current paper.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 for Windows (IBM, Amsterdam, The Netherlands).

Differences in distribution of categorical clinico-pathological parameters between groups were compared with χ^2 or Fisher's exact tests when appropriate.The distributions of RFS, CSS and OS were estimated using Kaplan–Meier methodology. For interpretation purposes analyses were stratified by tumour origin, that is, pancreatic head or periampullary. Univariate associations were tested using log-rank test. Cox proportional hazards regression model was used to test whether outcome measures were independent of other established prognostic factors (T status, nodal involvement and tumour differentiation). Besides these established prognostic factors, numbers were also corrected for adjuvant therapy to prove that results were independent of this potentially prognostic factor.

By the use of interaction terms it was investigated whether the prognostic effect of BM Laminin and collagen IV expression differed between pancreatic head and periampullary cancers.

All p-values reported are two sided and values $p \leq .05$ were considered statistically significant.

Results

Patient population

Of 231 tumours, 209 were adequately stained for analysis of BM laminin expression and 214 for analysis of BM collagen type IV expression. Both patient cohorts consisted of slightly more males than females, 119 vs 90 and 123 vs 91 in the laminin and collagen type IV cohorts, respectively. The median age of the patients in both cohorts was 65 years (range 36–87). Of 47% of tumours the origin was the pancreatic head, the other 53% were periampullary cancers. In both cohorts and for both tumour origins fewer patients had T1/2 tumours than T3/4 tumours, this was more pronounced in pancreatic head cancers (15% and 36% T1/2 tumours for pancreatic head and periampullary cancers, respectively). The amount of patients with lymph node involvement was approximately the same as those without lymph node involvement in both cohorts and for both tumour origins. Furthermore, in both patient cohorts and for both tumour types, the majority of tumours were moderately differentiated, with an equal amount of well and poorly differentiated tumours (on average 16%). Ten patients died during their postoperative stay and were thus excluded from survival analyses.

Basement membrane laminin and collagen type IV expression

Fifty-nine per cent of pancreatic head cancers showed <25% BM laminin, thirty per cent 25–75% and eleven per cent >75% BM laminin expression. Sixty-one per cent of periampullary tumours showed <25% BM laminin, thirty-three per cent 25–75% and six per cent >75% BM laminin expression. An example of BM laminin staining is given in Figure 1. Interestingly, positive intracellular staining of tumour cells was also observed.

In contrast, forty-three per cent of pancreatic head cancers showed <25% BM collagen type IV, forty-six per cent 25–75% and eleven per cent >75% BM collagen type IV expression. For periampullary cancer, expression rates were 41.4%,

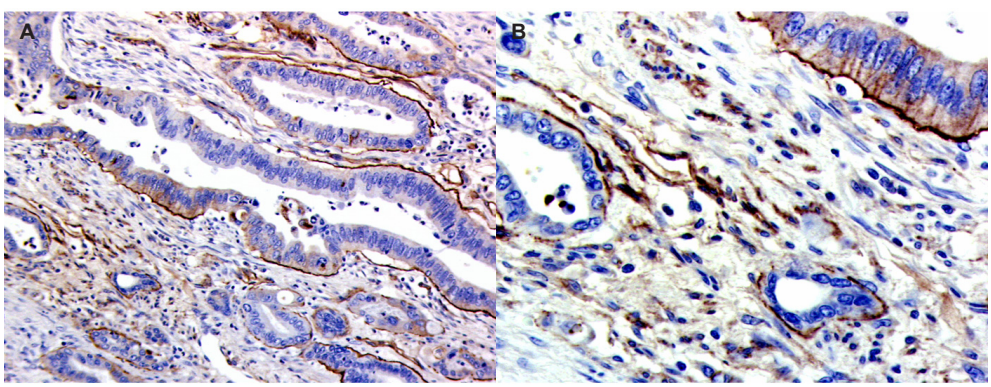

Fig. 1 – Pancreatic cancer staining with anti-laminin showing both basement membrane and tumour cell staining. (A) x10 magnification. (B) x20 magnification.

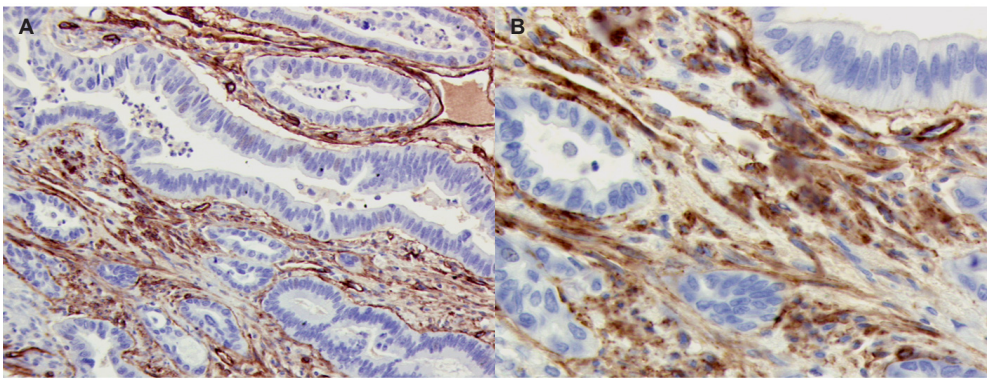

Fig. 2 – Pancreatic cancer staining with anti-collagen type IV showing both basement membrane staining and staining of tumour stroma. (A) x10 magnification. (B) x20 magnification.

47.4% and 11.2% for limited, 25–75% and >75% BM collagen type IV expression, respectively. Figure 2 is an example of BM collagen type IV staining. Apart from the BM, positive tumour stroma expression was also observed.
Furthermore, the graded levels of BM laminin and collagen type IV expression correlated weakly with each other (Spearman r=0.43 and 0.33 for pancreatic head and periampullary cancers, respectively, p< .001). In tumours with more discontinuous BM laminin staining, generally less BM collagen type IV staining was observed.

Pathologic correlations
Basement membrane laminin expression was not associated with any of the conventional prognostic factors (T or N status or grade of differentiation) in either tumour (i.e., pancreatic head or periampullary cancer). In contrast BM collagen type IV expression was associated with grade of differentiation in pancreatic head cancers (p= .037). A more fragmented BM collagen type IV expression pattern was observed in less differentiated tumours.

Clinical correlations
The pancreatic tumour types described, pancreatic head and periampullar cancer, showed significant different survival behaviour. Approximately two times as many patients were recurrence free and alive 5 years following complete resection of periampullary cancer as compared with pancreatic head cancer (29% vs 16%; p< .001 for all outcome measures).
To test whether tumour epithelial BM continuity, as determined by expression of its two major components laminin and collagen type IV, affects prognosis of tumours differently, depending on the tumour origin as specified above, we tested for effect modification (interaction) by tumour origin in the Cox models. This interaction test showed that the prognostic effect of BM laminin did not significantly differ between pancreatic head- and periampullary cancer. This was true for all

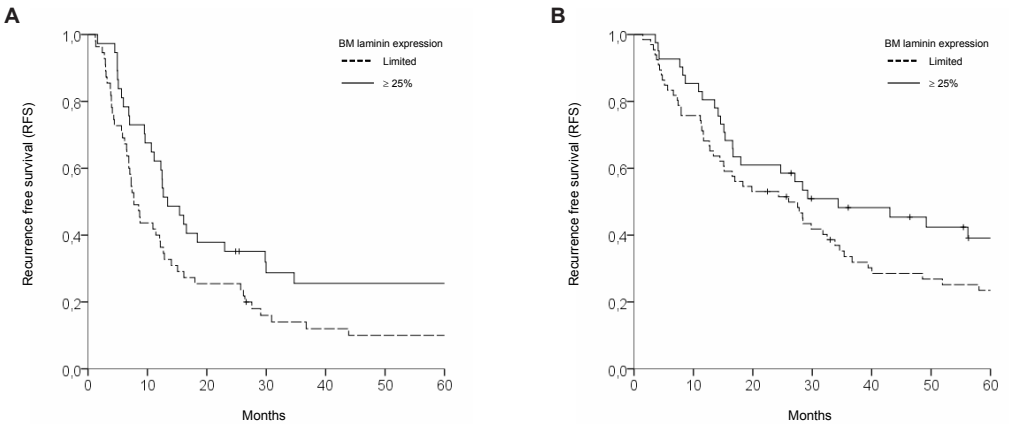

Fig. 3 – Recurrence free survival (RFS) of patients treated for, respectively, pancreatic head (A) and periampullary cancer (B) shows shorter RFS in patients with tumours with limited basement membrane (BM) laminin expression compared to those with tumours with more continuous BM laminin expression. This different survival behaviour, however, was only significant for pancreatic head cancers (p= .034 and .16 for pancreatic head and periampullary cancer, respectively).

outcome measures (p= .42, .19 and .35 for RFS, CSS and OS, respectively). Statistically, it would have been justified to take both tumours (i.e., pancreatic head and periampullary cancer) together for survival analysis. However, because of the significantly different survival of pancreatic head and periampullary cancer, both tumours were analysed separately for interpretation purposes.

Because of the relatively small amount of tumours classified as having >75% BM laminin (9 out of 92 and 7 out of 107 of, respectively, pancreatic head and periampullary cancer), both categories (25–75% and >75% BM laminin) were taken together as ≥25%. Survival analysis showed that BM laminin expression was significantly associated with outcome following curative resection of pancreatic head cancer (p= .034, .013 and .017 for RFS, CSS and OS, respectively). Ten per cent of patients treated for pancreatic head cancer showing <25% (i.e., limited) epithelial BM laminin were recurrence free and alive 5 years following curative resection of the tumour, whereas more than twice as many patients (26%) were recurrence free and alive if their tumours showed ≥25% BM laminin. A similar trend was observed for patients treated for periampullary cancer (24% and 39% for limited and ≥25% BM, respectively), however, this difference in survival behaviour was not significant (p= .16; .27 and .15 for RFS, CSS and OS, respectively). (Fig 3) By multivariate analysis it was shown that limited BM laminin expression is an independent predictor of poor OS and CSS following curative resection of pancreatic head cancer. This was not the case for RFS. When corrected for other prognostic factors such as tumour extent (T status), nodal involvement, grade of differentiation, BM laminin expression did however show a trend for an independent association with RFS (p= .09) (Table 1). Even when corrected for the adjuvant treatment given to some patients, BM laminin expression remained a signifi-

Table 1 – 5-Year survival and multivariate analysis of conventional prognostic factors and tumour BM laminin expression and outcome following curative resection of pancreatic cancer

Factor	N	RFS				CSS				OS			
		%5yr	HR	95% CI	p	%5yr	HR	95% CI	p	%5yr	HR	95% CI	p
Tumour extension					.30				.18				.39
T 1/2[a]	14	29				31				29			
T 3/4	76	13	1.41	0.74-2.69		17	1.67	0.78-3.56		14	1.33	0.69-2.55	
Nodal involvement					.024				.006				.006
No[a]	43	30				35				32			
Yes	49	3	1.75	1.08-2.86		3	2.10	1.24-3.54		2	1.98	1.22-3.22	
Differentiation					.09				.08				.10
Well[a]	15	33				38				33			
Moderately	60	12	1.99	1.04-3.80	.037	15	1.84	0.89-3.83	.10	13	1.97	1.03-3.76	.04
Poorly	16	13	2.12	0.98-4.59	.056	13	2.69	1.14-6.36	.024	13	2.02	0.93-4.41	.08
BM Laminin expression					.09				.043				.05
Limited[a]	55	10				11				10			
> 25%	37	26	0.67	0.42-1.07		31	0.59	0.35-0.98		27	0.63	0.39-1.00	

Abbreviations: BM = basement membrane; 95% CI = 95% confidence interval; CSS = cancer-specific surival; HR = hazard ratio; OS = overall survival; RFS = recurrence-free survival; % 5 year = % 5 year survival by univariate analysis. [a] Reference category.

cant prognostic factor for CSS and showed a trend for a significant association with RFS and OS (p= .11 and .058, respectively).
In contrast to BM laminin, BM collagen type IV expression was not associated with outcome in either type of pancreatic cancer (p= .35, .19 and .35; and .94, .89 and .85 for RFS, CSS and OS of pancreatic head- and periampullary cancer, respectively) (data not shown).

Discussion

This is the largest study to date studying the expression of BM components in pancreatic cancer specimens and investigating its potential relation with prognosis following curative resection.
In the current study, approximately one-tenth of pancreatic head and periampullary tumours showed ≥75% tumour epithelial cell lining by BM major components laminin or collagen type IV. This could be either due to increased turnover by proteolytic enzymes or decreased synthesis. In the 1980s, Liotta et al already showed that the rate of spontaneous metastases correlated with collagen type IV degradation activity in cells. Since then several matrix metalloproteinase's have been identified. In the current study some tumour cells showed immunoreactivity

for laminin, whereas immunostaining for collagen type IV was also observed of tumour stroma. Accumulation of collagen type IV in the interstitium was also described by Kalluri (2003) in association with fibrosis.

In line with the concept that synthesis and modulation of BM components have a major role in morphogenesis (Martinez- Hernandez and Amenta, 1983), BM collagen type IV expression was associated with tumour differentiation of pancreatic head cancers in our patient cohort. The more patchy BM collagen type IV staining, the least differentiated the tumour. In contrast, tumour differentiation was not associated with BM laminin expression. Both poorly differentiated tumours and well-differentiated tumours showed limited BM laminin expression, the same was true for tumour extent, an observation that suggests that disruption of BM laminin is an early process in tumour progression. The correlation of BM continuity with differentiation is in line with observations in bladder-, colorectal-, hepatocellular-, breast-, endometrial cancer and an earlier report on pancreatic cancer (Albrechtsen et al, 1981; Forster et al, 1984, 1986; Stenback et al 1985; Mollenhauer et al, 1987; Schapers et al, 1990; Grigioni et al, 1991; Lazaris et al, 2003; Souza et al, 2007). BM continuity has also been associated with stage in some tumours (Havenith et al, 1988; Schapers et al, 1990) and metastasis in others (Forster et al, 1984; Forster et al, 1986; Mielcarek-Kuchta et al, 2008).

As could have been expected by its highly crosslinked structure (Kalluri, 2003), BM laminin expression was associated with BM collagen type IV expression. Generally, tumours with limited BM laminin deposits also showed scarce collagen type IV expression.

Although widely fragmented BMs have been observed in pancreatic cancer before (Lee et al, 1994; Imamura et al, 1995; Shimoyama et al, 1995), there is only one small study of 16 patients analysing several extracellular matrix proteins and integrins stating that staining patterns were comparable irrespective of patient survival (Linder et al, 2001). The lack of an association between the BM patterns and outcome observed by Linder and coworkers could be due to the small patient sample. In contrast, in our study more than twice as many patients were recurrence free and alive 5 years following resection if their tumours had ≥25% BM laminin expression compared with patients with tumours with <25% BM laminin. In fact, limited BM laminin expression proved to be an independent predictor of poor survival following curative resection of pancreatic head cancer. Although BM laminin and BM collagen IV expression patterns were weakly correlated with each other, only BM laminin expression was associated with outcome. BM continuity by collagen type IV expression was not associated with outcome. BM by laminin was also associated with outcome in colorectal- (Forster et al, 1984; Forster et al, 1986; Lazaris et al, 2003), bladder- (Schapers et al, 1990) and hepatocellular cancer (Grigioni et al, 1991). In contrast to our findings a relation with BM by collagen IV was also observed in several tumours (Daher et al, 1987; Havenith et al, 1988; Schapers et al, 1990; Grigioni et al, 1991; ten Velde et al, 1991; Lazaris et al, 2003).

There are two mechanisms, by which laminin is thought to be involved in the formation of metastases. First, laminin has been reported to be involved in the for-

mation of hemidesmosomes, biological structures that enable static cell adhesion. Consequently, decreased expression could cause disassembly or a reduction in the number of hemidesmosomes, with failure of cell anchoring.
Second, the cleaved form of laminin has been observed to stimulate motility of epithelial cell types. Therefore, an increased expression of MMPs that cleave laminin could stimulate cell motility (Giannelli and Antonaci, 2000). A process in which activated pancreatic stellate cells might have an essential role (Schneiderhan et al, 2007).
The lack of a relation of collagen type IV expression with outcome in our study could theoretically have been caused by the fair-to-moderate agreement in scoring between the three observers. Apparently scoring BM collagen type IV expression is rather complicated. To draw definite conclusions with respect to the prognostic value of BM collagen type IV expression, other scoring systems need to be explored.
In conclusion, the current study is the first study that identifies an independent relationship between tumour BM laminin expression and prognosis of pancreatic head cancer. Routine tumour BM laminin staining could potentially differentiate between different prognostic subgroups of pancreatic head cancer and consequently aid in therapeutic decision making.

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Conflict of interest statement
The authors declare no conflict of interest.

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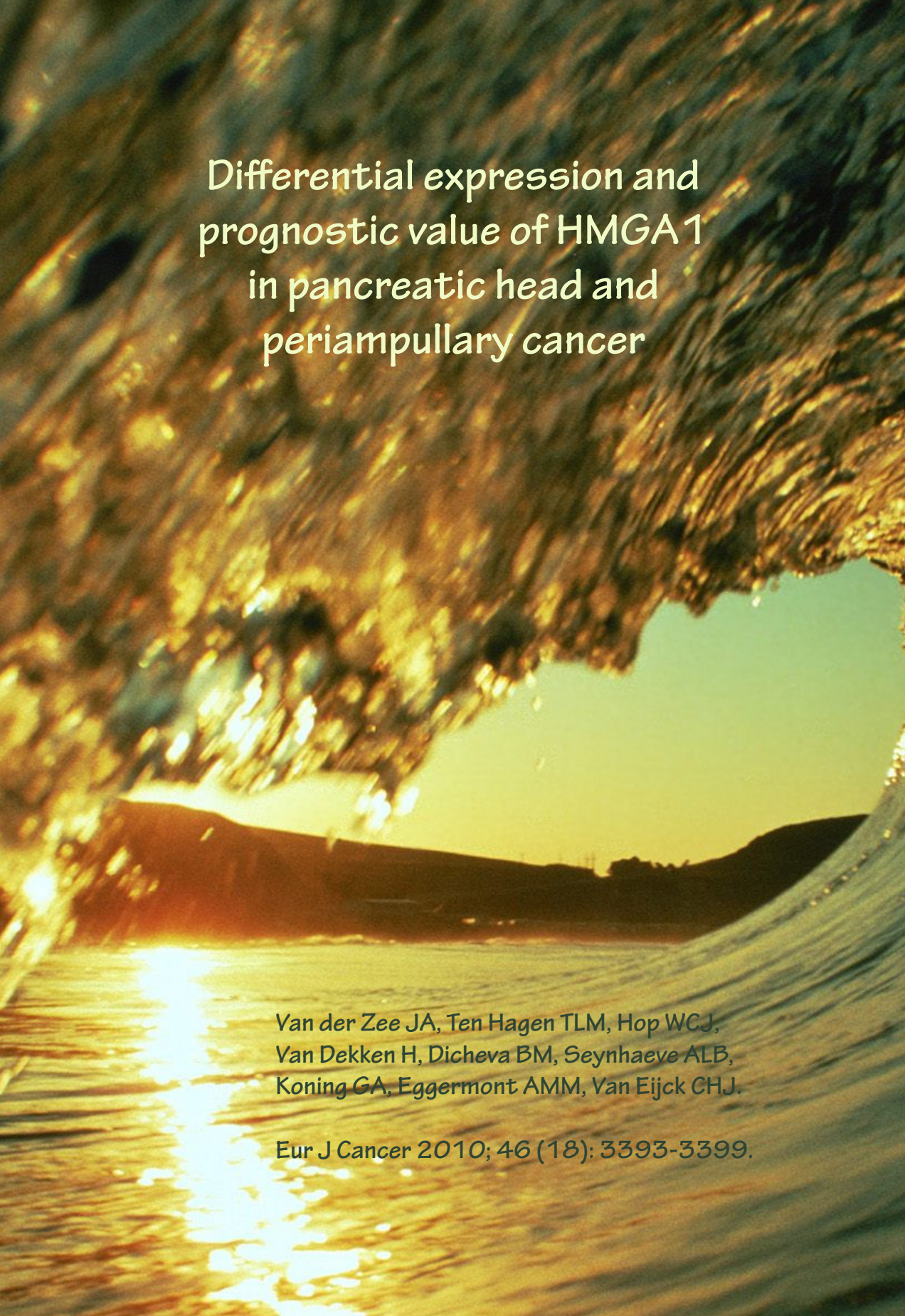

Differential expression and prognostic value of HMGA1 in pancreatic head and periampullary cancer

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Abstract

The high mortality rate and minimal progress made in the treatment of pancreatic cancer over the last few decades, warrant an alternative approach. Treatment protocols should be individualised to the patient guided by prognostic markers. A particularly interesting target would be the architectural transcription factor high mobility group A1 (HMGA1), that is low or undetectable in normal tissue, induced during neoplastic transformation and consequently often exceptionally high in cancer. The aim of the current study was therefore to determine the differential expression of HMGA1 in pancreatic head and periampullary cancer and investigate its relation with outcome.

HMGA1 expression was determined by immunohistochemistry on original paraffin embedded tissue from 99 pancreatic head- and 112 periampullary cancers (with R0).Expression was investigated for associations with recurrence free (RFS), cancer specific (CSS) and overall survival (OS) and conventional prognostic factors.

HMGA1 was expressed in 47% and 26% of pancreatic head- and periampullary cancer, respectively and associated with poor RFS, CSS and OS in periampullary cancer. CSS 5 years following surgery was 25% and 44% for patients with tumours which were positive or negative for HMGA1 protein, respectively. HMGA1 expression was not associated with survival in pancreatic head cancer.

In conclusion HMGA1 was identified as an independent prognostic marker predicting poor outcome in periampullary cancer. Although expressed to a higher extent as compared to periampullary cancer, HMGA1 was not associated with survival in pancreatic head cancer.

Introduction

With yearly mortality rates equalling incidence and a 5-year survival rate following diagnosis of 5%, pancreatic cancer remains a serious health problem.[1] Almost 80% of patients present with major vessel involvement or distant metastasis, precluding them from resection, currently still the only potential for cure. Even following resection most patients will develop recurrent disease.[2] Despite attempts to improve outcome by several adjuvant chemo- and/or radio-therapy regimens, little to no progress has been made in the last few decades.[3] Specialists therefore claim that therapy should be individualised to the patient, guided by prognostic markers. One such candidate marker could be high mobility group A 1 (HMGA1). HMGA1 proteins, originally discovered in HeLa cells,[4] are nuclear DNA-binding proteins which by interacting with the transcription machinery and altering chromatin structure, regulate the transcription of a multitude of genes.[5,6] HMGA1 has three isoforms encoded by the same gene, however generated through an alternative splicing mechanism.[7,8] HMGA1 is increased during embryogenesis and becomes low or undetectable in normal adult tissue.[9] However, increased levels have also been observed in rat thyroid cell lines following transformation with oncogenes.[10,11] Furthermore, neoplastic transformation was associated with HMGA1 expression in human prostate-,[12,13] thyroid-,[14,15] colorectal-,[16–20] cervix-,[21] pancreas-,[22–24] gastric-,[25] ovary-,[26] breast-,[27] liver-,[28] lung-,[29] uterine-[30] and head and neck-[31] tissue and blood.[32] A relation with worse pathological factors was observed in some of these.[12,13,18,20,28,33] Interestingly, following orthotopic injection of human pancreatic cancer cells, increased HMGA1 expression was observed in metastasis as compared to the primary tumour. A relation of HMGA1 expression with disease progression[13,20,31] and poor survival[24,28,29] was observed in some clinical studies. Of interest as well is the differential expression of HMGA1 between different neuroblastic tumours and different testicular germ cell tumours.[34,35]
The expression of HMGA1 proteins in cancer cells and not in their normal counterparts makes it a particular interesting target for therapy. Adenovirus mediated suppression of HMGA1 inhibited cell growth in carcinoma cells derived from human thyroid, lung, colon and breast, however had no effect on normal thyroid cells in vitro. Furthermore, adenovirusmediated suppression of HMGA1 in vivo reduced thyroid tumour size.[36] This growth inhibitory effect mediated by the suppression of HMGA1 was confirmed in pancreatic cancer.[37,38] Liau and co-workers showed that silencing of HMGA1, decreased anoikis resistance and cellular-invasiveness in vitro, metastatic potential in vivo and increased sensitivity to gemcitabine both in vitro and in vivo.[38–40] They also provided evidence suggesting HMGA1 to be an independent predictor of poor postoperative survival in patients with pancreatic adenocarcinoma.[24] The evidence for an important role of HMGA1 proteins in tumour progression and the differential expression between subtypes of some tumours, prompted us to investigate the differential expression of HMGA1 in pancreatic head and the prognostically more favourable periampullary cancer and explore the relation with the outcome in both tumour types.

Patients and methods

Patient population

Retrospectively, 231 patients treated for pancreatic adenocarcinoma with curative intend at Erasmus Medical Center between 1987 and 2008 who had no microscopically residual tumour (R0) were identified. Tumours were classified by location having its origin either in the pancreatic head or periampullary region, the latter group comprising tumours originating in the Ampulla of Vater or the distal common bile duct. Tumour samples originating before the new 2002 UICC TNM classification were re-evaluated according to these new criteria.

Representative tumour areas were encircled on original haematoxylin/eosin slides by a GI pathologist (HvD) with special expertise in pancreatic pathology and staining was performed on corresponding formalin fixed, paraffin embedded tissue.

During the above-mentioned period two randomized control studies were ongoing in our centre. Between September 1987 and April 1995, 17 patients were randomized to the treatment arm of the EORTC 40891 trial, receiving two courses of 5-FU as a continuous infusion (max 1500 mg/day) followed by radiotherapy (20 Gy). From June 2000 up to its closure in March 2007, 32 patients were randomized to the treatment arm of a trial combining intra-arterial chemotherapy and radiotherapy. Patients received six cycles of intra-arterial mitoxantrone (10 mg/m^2), folinic acid (170 mg/m^2/day), 5-FU (600 mg/m^2/day) and cisplatinum (60 mg/m^2), the first cycle followed by radiotherapy (54 Gy). These trials and the results have been described in detail elsewhere.[41,42] At the time of the present report, the median follow-up duration was 19 months (range 0–192 months). Recurrence free survival (RFS) was defined as the time from resection to first proof of disease recurrence (locally, distant or both) or to death without relapse. Overall survival (OS) was computed as the number of months from resection to death of any cause as registered by the social security death index (SSDI), whereas for cancer specific survival (CSS) only the pancreatic cancer related deaths were counted. Patients who died in hospital following procedure related complications were excluded from analysis with respect to survival as we consider their death to be unrelated to the studied tumour biology and would have introduced a confounding influence on survival analysis.

HMGA1 expression by immunohistochemistry

Immunohistochemistry was performed according to the protocol used in clinical practice at our institution and was optimised for HMGA1. Briefly, 4 µM sections were deparaffinized in xylene and rehydrated through decreasing ethanol series ending in distilled water. Antigen retrieval was performed by microwave heating (20 min preheating followed by 20 min of cooking) in Tris–EDTA buffer pH 9.0. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide (H_2O_2) in PBS for 20 minutes. Sectionswere incubated overnight at 4°C with a polyclonal mouse antibody to the full length HMGA1 protein (B01, Abnova Corporation Taipel, Taiwan) at 500x dilution in Dako REAL antibody diluent (S2022,

Dako), which reduces background staining without any need for additional block ing steps. This was followed by incubation with the secondary antibody (Dako REAL Envision HRP Rabbit/Mouse) for 30 min at RT. Immunostaining was developed by immersion in diaminobenzidine. Slides were washed extensively between each of the above steps. Nuclei were counterstained with Harris Haematoxylin, followed by dehydration, fixation and finally covered using Leica multistainer and robotic coverslipper (ST5020 and CV 5030, Leica Microsystems B.V., Rijswijk, Netherlands). Positive and negative controls were included in each run.

Tissue evaluation

Slides were examined and scored separately by three observers (J.A.v.d.Z.; B.M.D. and T.L.M.t.H.) blinded to both clinical and pathological data. HMGA1 expression was quantified using a visual grading system based on the extent of staining. Immunoreactivity in the nucleus was evaluated. HMGA1 was absent (<10% nuclei from ductal cells positive), present in low quantities (≥10% and <50% nuclei positive) or present in high quantities (≥50% positive nuclei) in the nuclei of tumour cells. Discrepant scores were resolved by consensus.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 for Windows. Differences in distribution of categorical clinico-pathological parameters between groups were compared with Chi-square or Fisher's exact tests when appropriate. The distributions of RFS, CSS and OS were estimated using Kaplan Meier curves. Univariate associations were tested using the Log-rank test. Cox- regression models were used to test if relations between HMGA1 expression and outcome were independent of other established prognostic factors (T status, nodal involvement and tumour differentiation). By the use of interaction terms it was investigated whether prognostic effects of HMGA1 differed between pancreatic head and periampullary cancers. All p values reported are two sided and values ≤ .05 were considered statistically significant.

Results

Patient population

Tissue blocks were available from 222 out of 231 patients. Eleven slides could not be scored due to poor quality. As a result immunostaining was correlated with established prognostic factors for 211 cases. The age of the patients ranged from 36 to 87 years (median 65 years) and the study population included slightly more males than females (122 vs 89). Ninety-nine tumours originated in the head of the pancreas whereas 112 patients had periampullary cancer. Nine patients died during postoperative stay and were thus excluded from survival analyses.

High mobility group A1 (HMGA1) expression

HMGA1 was heterogeneously expressed between and within tumours (Fig. 1). Staining was predominantly nuclear; however some perinuclear granulation was also observed. Since relatively few patients showed high HMGA1 expression (11 and 6 patients for respectively pancreatic head and periampullary cancer), low and high expression were combined for further analysis. Nuclear immunostaining was present in 47% (46/99) of pancreatic head tumours and 26% (29/112) of

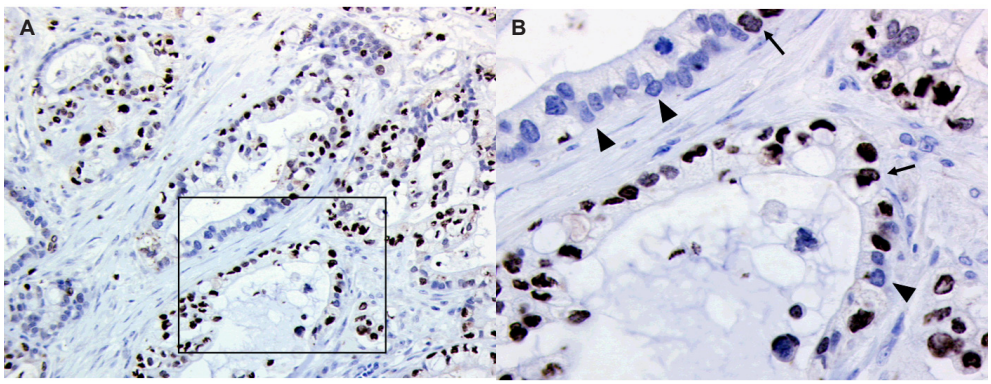

Fig. 1 – Periampullary cancer tissue immunohistochemically stained with HMGA1 antibody. (A) The tumour shows heterogeneous expression of HMGA1 (10x magnification). (B) Magnification of area depicted in (A) clearly showing both positive (arrow) and negative (arrowhead) nuclear staining (40x magnification).

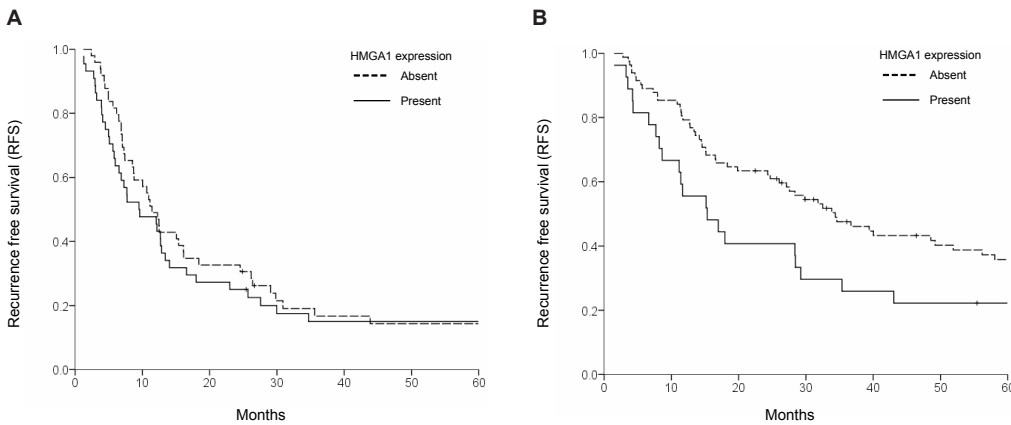

Fig. 2 – Kaplan Meier curves of recurrence free survival (RFS) of patients treated for respectively pancreatic head (A) and periampullary cancer (B) show a trend for shorter RFS in patients with periampullary tumours expressing HMGA1 compared to those with tumours that lack HMGA1 (p= .053). No difference in RFS was observed for patients with pancreatic head cancer (p= .36).

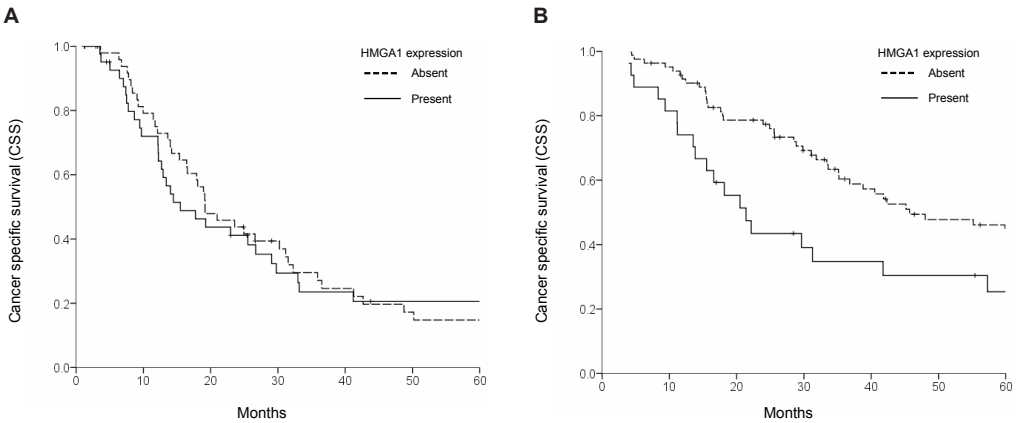

Fig. 3 – Kaplan Meier curves of cancer specific survival (CSS) of patients treated for respectively pancreatic head (A) and periampullary cancer (B) show shorter CSS in patients with periampullary cancer expressing HMGA1 compared to patients with tumours lacking expression (p= .019). No difference in CSS was observed for patients with pancreatic head cancer (p= .80).

periampullary tumours (p= .003).

Pathologic correlations

HMGA1 expression was not associated with any of the conventional prognostic factors in the total group of pancreatic cancers (p= .29, .24 and .28 for respectively T-, N- and differentiation status). Analysing pancreatic head and periampullary cancer separately gave similar results.

Clinical correlations

Patients with pancreatic head cancer had considerably worse prognosis following curative resection when compared to patients treated for periampullary cancer (p< .001 for RFS, CSS as well as OS). Only 17% of pancreatic head cancer patients were alive 5 years following curative resection as compared to 40% of patients treated for periampullary cancer. Survival analyses with respect to HMGA1 expression were therefore separated for the two tumour types.

In univariate analysis the presence of HMGA1 was significantly associated with cancer specific (p= .019) and overall survival (p= .017) and showed a trend for an association with recurrence free survival (p= .053) in periampullary cancer (Figs. 2–4). No significant associations were found in pancreatic head cancer (p= .91, .56 and .93 for respectively RFS, CSS and OS). Following correction for other conventional prognostic factors such as tumour extension, nodal involvement and degree of differentiation, HMGA1 expression proved an independent prognostic factor predicting a poor outcome following curative resection of periampullary can-

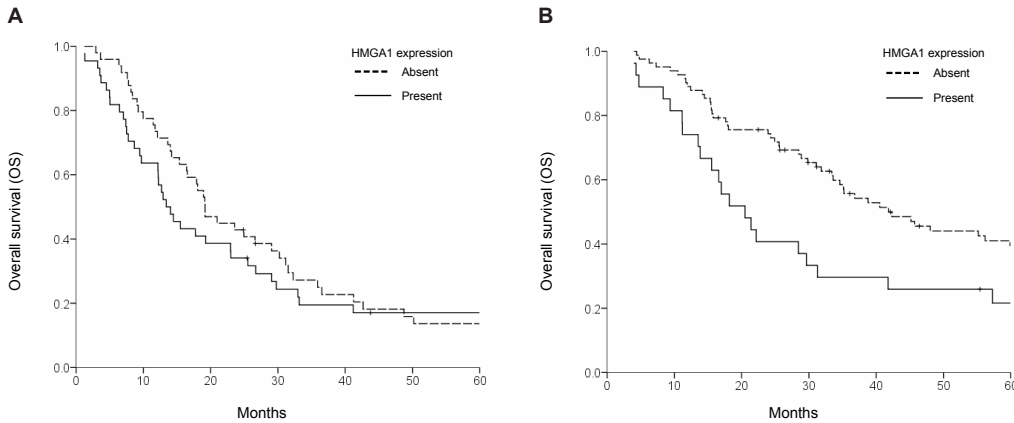

Fig. 4 – Kaplan Meier curves of overall survival (OS) of patients treated for respectively pancreatic head (A) and periampullary cancer (B) show shorter OS in patients with periampullary cancer expressing HMGA1 compared to those with tumours that lack HMGA1 (p= .017). No difference was observed for patients with pancreatic head cancer (p= .30).

cer (Table 1). Multivariate analysis in pancreatic head cancer also showed that HMGA1 protein levels had no effect on outcome (Table 2).
Further analysis showed that the adjusted HR for CSS in Tables 1 and 2 differed significantly between the two tumour types (HR= 2.01 versus 0.86 for periampullary and pancreatic head cancer respectively; p= .034). For the other two outcome measures however, the adjusted HR did not significantly differ between the two tumour locations (p= .14 and .11 for, respectively RFS and OS).

Discussion

This is the first study to date reporting the differential expression of HMGA1 in pancreatic head and periampullary cancer.
Our results are obtained in the largest series of pancreatic cancer studies thus far and in contrast to some of the prior reports: Abe and co-workers showed that all 15 pancreatic duct cancers investigated were positive (that is at least 20% of nuclei positive) for HMGA1, whereas this protein was absent in nuclei of normal pancreas cells.[22] Liau and coworkers observed a 93% positive rate for HMGA1 with a cut-off level of 5%.[24] There is no consensus on the definition of positive with respect to HMGA1 immunoreactivity, multiple cut-off levels have been reported in literature. We therefore decided to take a cut-off level in between the 20% and the 5% used by Abe and Liau, respectively. With a cut-off level of 10%, forty-seven and 26% of respectively pancreatic head and periampullary cancers were identified positive for HMGA1 in our study cohort. An explanation for the different ex-

Table 1 – Multivariate analysis periampullary cancers.

Factor	N	RFS				CSS				OS			
		%5yr	HR	95% CI	p	%5yr	HR	95% CI	p	%5yr	HR	95% CI	p
Tumour extension					.12				.16				.09
T 1/2[a]	40	52				56				53			
T 3/4	67	20	1.50	0.90-2.50		30	1.52	0.85-2.71		25	1.56	0.93-2.62	
Nodal involvement					.001				.003				.012
No[a]	51	51				56				51			
Yes	58	16	2.28	1.41-3.69		25	2.33	1.34-4.04		21	1.87	1.15-3.04	
Differentiation					.024				.072				.13
Well[a]	18	58				71				64			
Moderately	72	30	1.11	0.56-2.18	.77	37	1.34	0.59-3.06	.49	32	1.25	0.62-2.52	.54
Poorly	19	16	2.41	1.09-5.34	.031	23	2.53	1.00-6.42	.051	21	2.14	0.94-4.88	.07
HMGA1					.031				.015				.018
Absent[a]	82	36				44				40			
Present	27	22	1.77	1.05-2.96		25	2.01	1.15-3.53		22	1.18	1.12-3.17	

[a] Reference category.

Table 2 – Multivariate analysis pancreatic head cancers.

Factor	N	RFS				CSS				OS			
		%5yr	HR	95% CI	p	%5yr	HR	95% CI	p	%5yr	HR	95% CI	p
Tumour extension					.24				.23				.34
T 1/2[a]	13	23				25				23			
T 3/4	78	13	1.50	0.77-2.92		16	1.59	0.75-3.39		14	1.39	0.71-2.72	
Nodal involvement					.016				.003				.004
No[a]	44	27				31				28			
Yes	49	3	1.78	1.11-2.86		3	2.15	1.30-3.55		2	2.01	1.25-3.23	
Differentiation					.08				.042				.08
Well[a]	15	33				38				33			
Moderately	60	10	1.96	1.02-3.75	.043	13	1.89	0.92-3.92	.09	11	1.92	1.01-3.66	.048
Poorly	17	12	2.27	1.06-4.86	.034	12	2.96	1.27-6.86	.012	12	2.25	1.05-4.82	.037
HMGA1					.91				.56				.93
Absent[a]	49	14				15				14			
Present	44	15	0.98	0.62-1.52		21	0.86	0.53-1.41		17	1.02	0.65-1.60	

[a] Reference category.

pression levels observed in our study cohort when compared to the ones reported by Abe and Liau is not obvious. With respect to differences in patient popula-

tions, in our patient cohort fewer patients presented with nodal involvement when compared to the group described by Liau and co-workers, however neither study showed a significant relation of HMGA1 with the presence or absence of positive lymph nodes (p= .20 and .08 in respectively theirs and our cohort). Furthermore, although another antibody was used to stain for HMGA1, the antibody used in the current study recognises both HMG-R and HMGIY. We initially performed immunostaining with three different antibodies, including the one used by Liau and co-workers, however satisfactory results were only achieved with the antibody currently used. In other cancers percentages ranging from 13% up to 95% were observed with the same cut-off used by us.[17,26–28,31,43] The different expression levels observed in pancreatic head and periampullary cancer are in line with previous studies in neuroblastoma and testis, showing different expression levels in the different subtypes of these cancers.[34,35]

We observed that besides different expression levels between the two types of pancreatic cancer, HMGA1 was identified as an independent predictor of outcome in only one of the two cancer types. Lack of HMGA1 was associated with increased relapse free-, cancer specific- and overall survival in periampullary cancer. Five year cancer specific survival rates were 25% and 44% for patients with tumours respectively positive and negative for HMGA1 protein. HMGA1 expression did not determine outcome in pancreatic head cancer. This is in contrast to the observation made by Liau and co-workers. They observed a 12 times higher risk of death in the 93% of patients with tumours positive for HMGA1 compared to the remaining 7% lacking HMGA1 expression in their tumours when adjusted for age, gender, tumour size and differentiation, lymph node status and lymphovascular invasion.[24] The different expression levels of HMGA1, but even more so the information that the relation of HMGA1 with prognosis is restricted to periampullary cancer, suggest that these two tumour types not only differ with respect to prognosis, but also clearly seem to have different molecular behaviour. This is strengthened by similar observations for two other tumour markers, Bag-1 and TS, conversely for these the prognostic effect was restricted to pancreatic head cancer (data not shown).

In conclusion, in the current study the multifunctional architectural transcription factor HMGA1 proved to be an independent marker predicting poor survival in periampullary cancer. The lack of expression in normal tissue makes HMGA1 a particularly interesting target for therapy. The absence of an association with outcome in pancreatic head cancer makes HMGA1 a less interesting target for the treatment of this type of pancreatic cancer.

Conflict of interest statement

The authors declare no conflict of interest.

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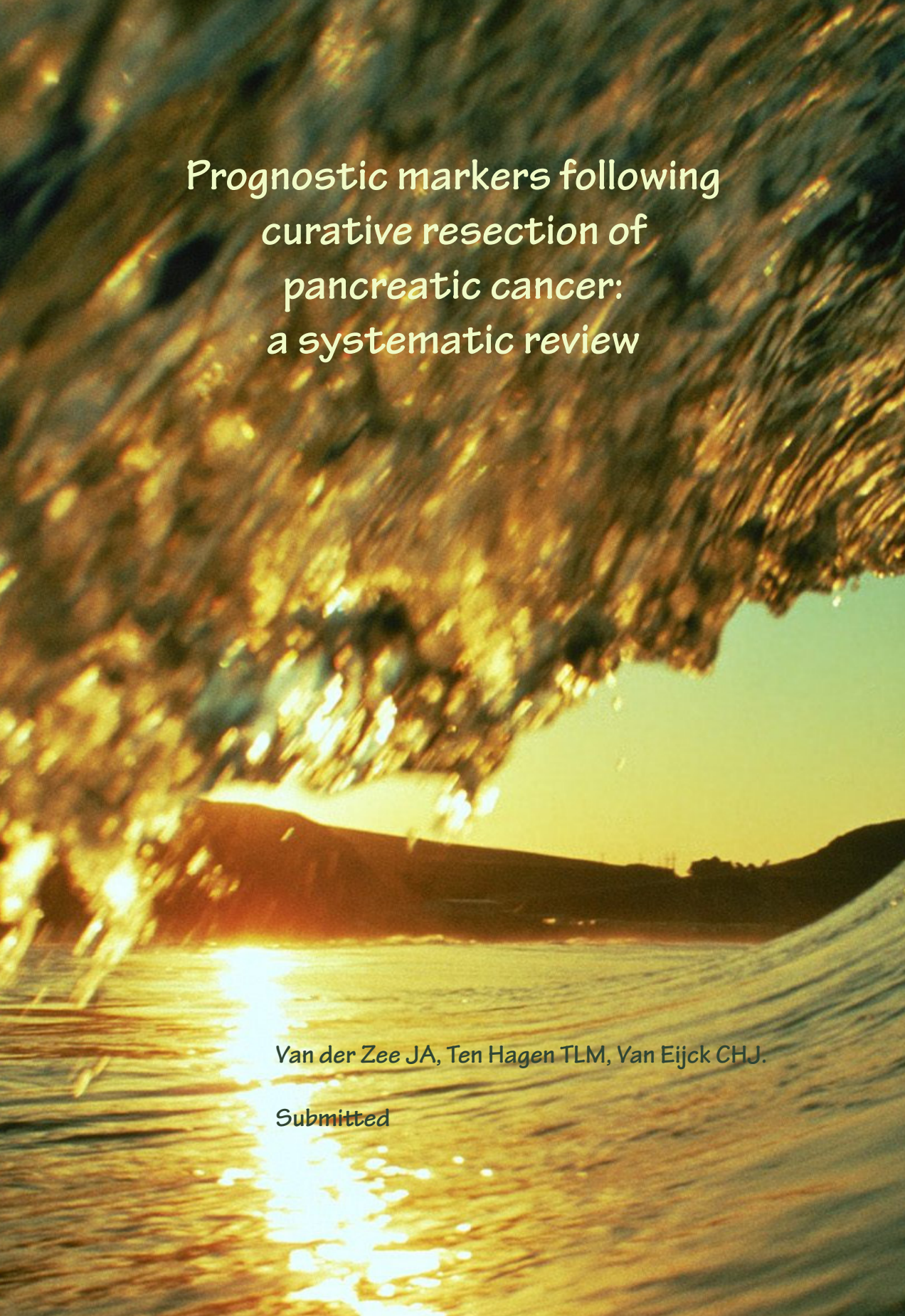

Prognostic markers following curative resection of pancreatic cancer: a systematic review

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Submitted

Abstract

Pancreatic cancer has a dismal prognosis. Currently resection offers the only potential for cure. However even following curative resection, survival remains poor. Despite attempts to improve outcome by several adjuvant treatment regimens, survival has hardly improved over the last 20 years. Molecular markers identifying certain prognostic subgroups, could aid in therapeutic decision making or even become targets of their own. Unfortunately, most prognostic studies include both localized and advanced disease, clearly representing different prognostic situations, hampering interpretational value.

The current systematic review therefore aimed to identify a set of proteins in pancreatic and periampullary cancer independently predicting survival in a more homogeneous group of patients, exclusively including radical (R0) resections.

Seventeen immunohistochemical markers were identified by a Pubmed- and Embase search. Additionally three recently published markers of our own series were discussed. Proliferation markers Akt, HDGF, Ki67 and TS proved independent prognostic markers. Two tumour suppressors (AP-2α and PML) and three proteins involved in apoptosis (Bax, Bag-1 and VCP) were of prognostic value. While COX-2, involved in angiogenesis, was identified as a prognostic marker, the prognostic value of microvessel density remains inconclusive. With respect to tumour invasion and metastases ADAM9, CXCR4, DSC2, EpCAM, HMGA1, maspin, MUC2 and 17, pepsinogen and basement membrane component laminin were found to have a predictive value following curative resection.

Introduction

Pancreatic cancer is one of the most lethal forms of cancer with mortality rates approaching incidence.[1] In 2008 there were an estimated 278.684 new cases and 266.669 deaths from pancreatic cancer worldwide.[2]

Currently resection offers the only potential for cure. Unfortunately 80% of patients present with advanced disease, precluding them from resection. Even following curative resection, recurrence remains a major problem. Five year survival rates following resection are 20-25%.[3] In an attempt to improve patient outcome, several adjuvant treatment protocols were investigated.[4-10] Despite these efforts to improve outcome, unlike most of the more frequent causes of cancer mortality, whose death rates are declining, the death rate for pancreatic cancer has been relatively stable over the last 20 years.[2,11] Generally, therapeutic agents only benefit a subset of treated patients. The delineation of cancer phenotypes based on molecular markers of therapeutic responsiveness and overall outcome can enable stratification of patients to appropriate individualized therapeutic regimens, so unnecessary adverse side-effects of ineffective drugs are minimized.

Of the multitude of markers under investigation in pancreatic cancer only few were found to be of prognostic value.[12-16] Unfortunately, most studies comprise of very heterogeneous patient sets, including both radical and irradical, or even palliative resections, clearly representing different prognostic situations and consequently influencing interpretational value.

We therefore decided to perform a systematic review of all English literature on prognostic markers identified by immunohistochemistry in patients following curative resection of pancreatic and/or periampullary cancer.

Patients and methods

A computerized search of the PubMed and Embase databases was performed by the first author to identify all English-language papers on prognostic markers in pancreatic head and periampullary cancer. The following search terms were used for respectively Pubmed and Embase: (pancreatic[tw] OR pancreas[tw] OR distal common bile duct[tw] OR periampull*[tw] OR ampull*[tw]) AND (neoplasm*[tw] OR cancer*[tw] OR tumor[tw] OR tumors[tw] OR tumour*[tw] OR malign*[tw] OR carcinom*[tw]) AND (Biological Markers[mesh] OR marker*[tiab] OR biomarker*[tw] OR immunohistochemistry[mesh] OR Immunohistochem*[tw]) AND (prognosis[mesh] OR prognos*[tw] OR survival[tw]) NOT (animals[mesh] NOT humans[mesh]) NOT (editorial[pt] OR letter[pt] OR Practice Guideline[pt] OR review[pt] OR case reports[pt]) AND eng[la]; ((pancrea* OR ampull* OR periampull* OR 'distal common bile duct') NEAR/3 (neoplasm* OR cancer* OR tumor* OR tumour* OR malign* OR carcinom*)):de,ab,ti AND (Immunohistochemistry/exp OR Immunohistochem*:ab,ti OR marker*:de,ab,ti OR biomarker*:de,ab,ti OR marker/exp) AND (prognos* OR survival*):de,ab,ti NOT ([animals]/lim NOT [humans]/lim) NOT ([editorial]/lim OR [letter]/lim OR [review]/lim OR 'case report':ti OR [Conference Abstract]/lim OR [Conference paper]/lim OR [Conference re-

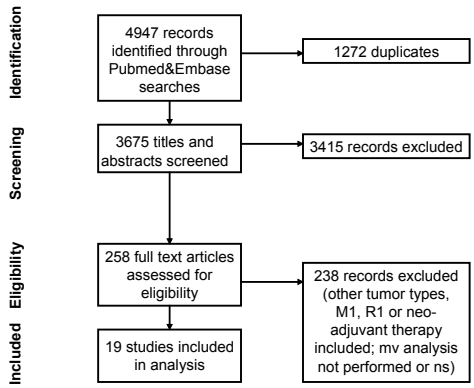

Fig. 1 – PRISMA flow diagram illustrating selection of articles for systematic review.

view]/lim) AND [English]/lim
The last update was performed on December 19th 2011.
The primary outcome measure was survival. Exclusively, studies including patients treated for pancreatic or periampullary cancer by curative (R0) resection were considered. Furthermore, only markers identified by immunohistochemistry and found to be of prognostic value independent of conventional prognostic factors by multivariate analyses, were included.
Existing systematic reviews and reference lists were also checked for any potential relevant additional studies. No attempt was made to acquire any missing data from investigators or sponsors.
Titles and abstracts were initially screened for meeting the inclusion criteria and the full text was retrieved for all potentially eligible studies. Data were extracted by the first author and reported on data extraction sheets. Of each study, the number of patients, the protein of interest, the Hazard ratio, the confidence intervals and p-value were recorded. The senior author checked the extracted data. Disagreements were resolved by consensus. Identified markers were categorized according to the recently updated hallmarks of cancer: sustaining proliferative signalling, evading growth suppressors, evading cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis.[17]
Reporting was done according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA).[18,19]

Results

The search of Pubmed and Embase databases provided a total of 4947 citations of which 1272 were duplicates, leaving 3675 papers. Of these, 3415 studies were

discarded during title- and abstract screening, while it appeared that these papers clearly did not meet the criteria. Two studies were discarded because the full text of the study could not be retrieved. The full text of the remaining 258 citations was examined in more detail. It appeared that 239 studies did not meet the inclusion criteria as described; consequently 19 studies describing 18 markers were included in the review. Figure 1 represents a flow diagram of the selection process. Additionally, three recently published markers of our own series are discussed. The study characteristics of the studies included are outlined in Table 1.

Table 1 – Characteristics of included studies.

Biomarker	Reference	Year	N	Hazard Ratio (95% CI)	p
ADAM9	[92]	2004	42	2.85 (1.21-6.71)	<.05
Akt	[22]	2004	61	3.44 (1.28-2.80)	.0015
AP2α	[38]	2010	63	5.412 (1.944-15.06)	.0012
Bag-1	[62]	2012	95	0.56 (0.33-0.98)	.041
Bax	[52]	2005	39	0.371 (0.210-0.863)	.020
BM by laminin	[118]	2012	92	0.63 (0.39-1.00)	.050
COX-2	[85]	2005	39	4.330 (1.257-9.569)	.005
CXCR4	[95]	2009	71	2.54 (1.27-5.10)	<.001
DSC2	[98]	2011	115	4.351 (1.857-10.085)	.0007
EpCAM/MK-1	[102]	2011	95	1.944 (1.15-3.28)	.013
	[101]	2006	38	68.27(2.79-1673.26)	.0097
HDGF (nucl)	[26]	2006	43	3.02 (1.18-8.65)	.0215
HMGA1	[106]	2010	109	1.88 (1.12-3.17)	.018
Ki67	[29]	1999	14	0.1574	.005
	[22]	2004	61	3.92 (1.20-3.72)	.0055
Maspin	[110]	2007	223	2.433	.0106
MVD	[76]	2002	45	6.5221	.0217
MVD, length, shape	[75]	2007	32	1.06 (1.02-1.11)	.009
				1.46 (1.04-2.03)	.027
				0.29 (0.10-0.80)	.016
MUC2	[113]	2009	59	0.426	0.047
MUC17	[38]	2010	63	42.07 (6.355-278.5)	.0001
Pepsinogen C	[117]	2001	67	3.22 (1.28-8.33)	.0137
PML (diffuse vs absent)	[43]	2009	62	0.359 (0.259-0.562)	.009 (overall p)
TS (high vs absent)	[32]	2012	91	0.31 (0.16-0.59)	<.001
VCP	[70]	2004	83	2.42 (1.11-2.26)	<.01

Sustaining proliferative signalling

The serine/threonine kinase Akt (also called Protein Kinase B) is best known for its role in proliferation. However, it also plays a central role in several other signalling cascades that regulate normal cellular processes, such as cell size, survival, and glucose metabolism. Additionally Akt is involved in sustained angiogenesis, unlimited replicative potential and tissue invasion and metastases. Three family members have been identified in mammals; Akt-1, Akt-2 and Akt-3 respectively. Akt activation requires membrane translocation and phosphorylation, which is triggered by growth factors and cytokines. Hyperactivation of AKT kinases is one of the most common molecular perturbations in human malignancy and has been reported in several cancers, including pancreatic cancer.[20,21] In fact, phosphorylated Akt was identified as an independent prognosticator in 61 patients who underwent curative resection of primary pancreatic ductal adenocarcinoma (PDAC) (HR 3.44; p= .0015). Patients with low intensity of p-Akt expression were observed to have better 5-year survival rates than those with high intensity expression, 57% and 14.1% respectively.[22]

In contrast, in another study including 39 pancreatectomy patients, Akt and p-Akt, were identified as favourable prognostic factors following resection. However this study included 3 patients with stage IV cancer that had microscopic evidence of peritoneal disease in the resected specimen.[23]

Hepatoma-derived growth factor (HDGF) is a member of the heparin binding growth factors that stimulates the proliferation of fibroblasts, endothelial cells, smooth muscle cells and neuronal cells.[24] HDGF is highly expressed in the early stage of organ development and thus considered closely related to ontogeny. HDGF translocates to the nucleus by nuclear localization signals, a process necessary for the induction of cell growth activity.[25] HDGF was identified as an independent prognostic marker in several tumours. With respect to pancreatic cancer, one study identified nuclear HDGF labelling index as an independent factor predicting poor survival following curative resection of 43 primary ductal carcinomas of the pancreas (HR 3.02; p= .0215). Five year survival rates were 37% and 6.8% for less and more than 90% expression, respectively.[26]

Ki-67 is a high molecular weight protein present in the nuclei of cells in the G1, S, and G2 phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G0 phase do not express the Ki-67 antigen. It is therefore claimed a marker of proliferation.[27,28]

The value of Ki-67 as a prognostic marker has been investigated in a wide range of studies, some identifying an independent association with prognosis, others that did not. Unfortunately, most studies analyzed patients with advanced disease or incomplete resections.[13] There were two studies identifying Ki-67 as an independent prognostic factor following curative resection of pancreatic cancer. The first, a relatively small (14 patients) study, described an association with stage, lymph node status, perineural invasion and poor outcome. The second, evaluating expression in 61 patients, confirmed the negative prognostic value of Ki-67.

5-Year survival rates were 66.3% and 18.8% for less and ≥20% expression, respectively (p< .05).[22,29]
Linder and coworkers evaluated 53 patients with stage I-III pancreatic cancer in whom curative resection was attempted, and found high proliferative activity by Ki-67, expressed as proliferating cell area (PCA), but not as proliferating cell index (PCI), to be of prognostic value.[30]

Thymidylate synthase (TS), well known as the primary target of 5-FU, is a rate limiting enzyme in the novel synthesis of DNA. In addition to its catalyzing function, TS functions as an RNA binding protein. Besides binding to its own mRNA and as such regulating its own biosynthesis, it interacts with several other mRNA's including those corresponding to p53 and the myc family of transcription factors. In these cases TS functions as a translational repressor, altering the cells capacity to respond to therapy or to survive and proliferate.[31]
We recently published TS to be of prognostic value in 91 curatively resected pancreatic head cancers. Patients with tumours lacking or showing low cytosolic TS expression were respectively 3 and 2 times more likely to die of pancreatic cancer than patients with tumours showing high expression. This prognostic effect proved independent of other established prognostic factors, such as T status, nodal involvement and tumour differentiation (p= .001).[32]

Evading growth suppressors
The activator protein-2 (AP-2), originally purified in 1987,[33,34] forms a family of six proteins, of which AP-2α, AP-2β and AP-2γ are the most well known. They are cell type specific DNA-binding transcription factors that regulate various signalling pathways. They are essential in development, cell growth, differentiation and apoptosis. Aberrant AP-2 activity has been associated with malignant transformation. A trend for a tumour suppressive role has been suggested in prostate cancer, colorectal carcinoma, melanoma, ovarian cancer and breast cancer and reduced expression was consequently associated with worse outcome in the latter three.[35] In pancreatic cancer AP-2α showed to efficiently repress proliferation and invasion of pancreatic cancer cells and increase sensitivity to gemcitabine.[36,37]
Recently, Hirono and coworkers performed gene expression profiling to identify genes associated with lymph node status in pancreatic ductal adenocarcinoma (PDAC). AP-2α was identified as one of the genes downregulated in patients with lymph node metastases. They validated their results at the protein level and then further confirmed their results in a validation set. Besides a significant association with lymph node metastases, AP-2α was identified as an independent predictor of favourable outcome in a set of 63 patients treated for PDAC by radical resection (HR 5.412; p= .0012).[38]

p53, recently celebrating its 30th anniversary, is one of the most frequently altered, and most intensively investigated genes in human cancer. It is part of a family of three mammalian proteins, the other two being p63 and p73. In addition, p53 itself produces nine isoforms by alternative splicing and transcriptional

initiation. The wild type protein acts as a tumour suppressor. Under physiological circumstances p53 level is maintained at a low level by a feedback loop. However various stress signals increase p53 protein expression resulting in transcriptional activation or repression of target genes, causing transient growth arrest in order to adapt to these unfavourable circumstances, or in case of severe stress, inducing replicative senescence or even apoptosis. P53 is either mutated or lost in most human tumours.[39,40] P53 expression and its prognostic value have been thoroughly investigated in pancreatic cancer, however most studies involved advanced disease or irradical resections. Some previous reviews, including a meta-analysis, have concluded that there is no relation between p53 and patient prognosis in pancreatic cancer.[12-16]
There was one study including stage I-III cancers for which curative resection was attempted, showing an independent association of p53 staining with poor survival (HR 3.26; p< .001). However no information was given on whether a radical resection was achieved.[30]

The t(15;17) translocation, specifically associated with acute promyelocytic leukaemia (PML), fuses the retinoic acid receptor α (RARα) locus to the PML gene.[41] Twelve PML isoforms, generated by alternative splicing of RNA, have been identified in humans. Most isoforms contain a nuclear localisation signal (NLS); the ones that lack a functional NLS are localized to the cytoplasm. One isoform contains a nuclear export signal, enabling shuttling between the nucleus and the cytoplasm. The variable C terminal is unstructured creating high flexibility, facilitating multiple interactions. By its interactions PML appears to cause cellular senescence and apoptosis. PML expression was shown to be lost not only in hematopoietic tumours, but also in solid tumours like breast-, gastric-, lung- and prostate cancer. Since mRNA levels are similar in cancer- and noncancerous tissues, expression is thought to be regulated by the ubiquitin/proteasome system following various cellular stress signals.[42]
PML was recently identified as a favourable prognostic marker following radical resection of 62 cancers of the ampulla by Vincenzi and coworkers. Median survival was 40, 48 and 77 months for absent, focal and diffuse PML expression (p= .002). Besides its prognostic value, PML was associated with T and N status and tumour grade.[43]

Another molecule drawing interest as a prognostic marker in pancreatic cancer is TGFβ.[13-16] TGFβ is a cytokine with a dual function. Under physiological circumstances TGFβ enforces homeostasis and suppresses tumour progression directly through cytostatis, differentiation and apoptosis, or indirectly through suppression of tumourigenic inflammation and suppression of stroma derived mitogens. However, when cancer cells lose TGFβ tumour suppressive responses, they can use TGFβ to initiate immune evasion, growth factor production, differentiation into an invasive and metastatic phenotype.[44-46]
Off all studies identifying TGFβ as a prognostic marker, only one could have potentially been eligible for our study. Nio and coworkers found TGFβ expression

to be a favourable characteristic in 91 patients with resectable invasive ductal carcinoma of the pancreas (RR 0.485; p= .0033). This study did however include 29 stage IV cancers.[47]

Evading cell death

Apoptosis or programmed cell death is a central regulator of tissue homeostasis. Apoptosis can be initiated by two alternative pathways. In type I cells the amount of initiator caspases is sufficient to induce executioner caspases directly, whereas in type II cells mitochondria are required as signal amplifiers.[48] Pancreatic cancer cells are type II cells and are consequently dependent on mitochondria for apoptosis. B-cell lymphoma-2 (Bcl-2) , originally identified in 1984,[49] plays a major role in this pathway. Several Bcl-2 family members have been identified, they can be divided in two groups; anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bax, Bak, Bad) proteins, where the antiapoptotic members preserve mitochondrial membrane integrity and pro-apoptotic members induce membrane permeabilization. Bcl-2 is best known for its ability to suppress apoptosis, however under certain circumstances it can undergo phenotypical conversion to an apoptosis inducer.[50,51]
Bcl-2 family proteins have been intensively investigated as prognostic markers in pancreatic cancer. Most studies included advanced disease. There is however one study that identified Bax as independent prognostic marker following radical resection in a cohort of 39 ampullary cancers (p= .020). Median survival was 120 months for patients with tumours showing high- and 28 months for those with tumours showing low expression.[52]
Although data are conflicting, a recent meta-analysis concluded that both Bax and Bcl-2 are associated with favourable survival.[12]

Bcl-2 associated anthanogen-1 (Bag-1), is a multi-functional protein that was identified in the search for novel interaction partners of Bcl-2.[53,54] It has four isoforms with different subcellular locations of preference, probably accounting for the wide range of proteins it interacts with. In addition to Bcl-2, Bag-1 was observed to associate with Hsp70, nuclear hormone receptors, RAF-1 kinase, components of the ubiquitylation/proteasome machinery and DNA. Consequently it is involved in apoptosis, cell signalling, stress-response/protein degradation, proliferation, and transcription.[55] Bag-1 has been observed to be overexpressed in cancer cells and has been associated with outcome in several cancer types.[56-61] Bag-1 isoforms are tissue specific, which might account for the variable effect of Bag-1 on outcome in different tumours, or even different subtypes of tumours as we found Bag-1 to be of prognostic value exclusively in pancreatic head cancer and not in periampullary cancer. Eighteen percent of patients with tumours showing nuclear Bag-1 were recurrence free and alive 5 years following curative resection, compared with none of the patients lacking expression (p= .003).[62]

Another apoptotic marker often evaluated in relation to pancreatic cancer prognosis is survivin.[13,14,16] Survivin, originally identified by Altieri,[63] is a member of the inhibitors of apoptosis (IAP) family. Survivin is expressed in embryonic and

fetal tissue and almost undetectable in adult tissue. In contrast, overexpression of survivin has been reported in almost all human malignancies, with increasing expression levels passing through the different stages of malignancy.[64] This accounts also for pancreatic carcinogenesis. While no expression has been found in normal pancreatic ducts, survivin expression steadily increased from low-grade pancreatic intraepithelial neoplasia to high-grade lesions and to the highest in pancreatic ductal adenocarcinoma tissues.[65] Of all studies performed in pancreatic cancer, only one was potentially eligible. Tonini and coworkers identified nuclear survivin as a marker of favourable outcome following macroscopic radical resection of pancreatic cancer, whereas cytoplasmic survivin was associated with poor outcome (RR 0.430, p=. 002; RR 0.556, p= .040 for nuclear and cytoplasmic survivin respectively).[66]

Valosin containing protein (VCP),[67] also known as p97, is a member of the type II ATPases associated with a variety of activities. It is one of the most highly evolutionary conserved proteins. VCP acts as a molecular chaperone that cooperates with the ubiquitin-proteasome system in regulating a wide variety of cellular processes, including protein degradation, membrane fusion, ER-associated degradation, transcription factor control, cell cycle progression and apoptosis. One of the transcription factors regulated by VCP is NF-κB.[68,69]
Yamamoto and co-workers found VCP to be associated with lymph node metastases and identified this protein as an independent marker of poor outcome following curative resection of pancreatic ductal adenocarcinoma in a cohort of 83 patients (HR 2.42; p< .01). Five-year survival rates were 59% and 21% for patients with low and high VCP expression respectively. It was hypothesized that its involvement in the NF-κB pathway might make the tumour cells resistant to immunologic attacks, allowing the cells to survive in lymph nodes.[70]

Inducing angiogenesis
Angiogenesis plays an important role in tumour growth and progression by supplying necessary oxygen, growth factors and nutrients, as well as by facilitating the haematogenous dissemination of tumour cells.[71-74] In an attempt to translate observations from experimental models to clinical practice, quantification of tumour vessels has been performed and correlations with clinicopathological factors and outcome have been investigated. Irrespective of the method used, the amount of tumour microvessels was associated with recurrent disease and poor survival in several primary tumours. Quantification of tumour microvessels has also been performed in some relatively small sized studies of pancreatic cancer; however the results with respect to its relation with prognosis are conflicting. We identified two studies clearly fulfilling our selection criteria. Giannopoulos and coworkers investigated both the quantity and quality of microvessels in 51 resected pancreatic ductal and ampullary cancers and found both to be associated with N1 disease and prognosis in pancreatic ductal carcinoma (p= .009, .027 and .016 for MVD, minor axis length and shape factor, respectively).[75] Stipa and coworkers also identified microvessel density (MVD) as an independent predictor of out-

come in 45 radically resected pancreatic head cancers. Median survival was 34.3 months and 15.08 months for the hypovascular and hypervascular subgroups, respectively (p< .0001).[76]
Another group performed pancreaticoduodenectomy with curative intent in 22 patients with ductal adenocarcinoma of the pancreas. They identified vascular surface density (VSD) as an independent predictor of poor outcome, whereas number of vessels per mm^2 stroma (NVES) was of borderline importance (HR 1.88 and 1.129; p= .039 and .056 for VSD and NVES, respectively).[77] Linder and coworkers attempted curative resection in a set of 45 patients and found the location of the hot spots (i.e. restricted to stroma only or neoplastic parenchyma) to be of prognostic value (p= .006).[78] IMD was found to be a valuable tool in predicting relapse free and overall survival by Fujioka and coworkers in 104 patients with primary pancreatic cancer of which 8 patients had positive para-aortic lymph nodes, treated by surgery with regional lymph node dissection (p= .019).[79]
Our own group, however recently published a study showing no association of tumour angiogenesis with survival in 206 curatively resected pancreatic head- and periampullary cancers.[80] The prognostic value of angiogenesis therefore remains unclear, as was also previously concluded in another review.[13]

Cyclo-oxygenase-2 (COX-2),[81,82] also known as prostaglandin H2 synthase-2 (PGHS-2), is an enzyme that catalyzes the initial step in the oxidation of arachidonic acid to the prostanoids. It is undetectable in most normal tissues, but can be induced by inflammation, mitogens, cytokines and growth factors. COX-2 is involved in several oncogenic processes, including apoptosis inhibition, continuous growth, induction of angiogenesis, invasion and metastasis, evasion of the immunologic anti tumour response and the activation of carcinogens. Most of these seem to be driven by prostaglandin E2 (PGE2), a major downstream effector of COX-2.[83,84] One study in 39 ampullary cancers, identified COX-2 as an independent predictor of poor survival following resection without residual disease. Median survival was 16 versus 73 months in patients with high and low COX-2 expression respectively.[85]
Although no additional benefit was shown in a phase II study of celecoxib, a selective COX-2 inhibitor, in addition to gemcitabine and cisplatin for patients with advanced pancreatic adenocarcinoma,[86] a recent case control study from Mayo Clinic observed a significantly reduced risk of pancreatic cancer with the monthly use of aspirin.[87]

Activating invasion and metastasis
The ADAMs (a disintegrin and metalloproteinase) are a family of transmembrane and secreted proteins that regulate cell phenotype through their effects on cell adhesion, migration, proteolysis and signalling. Of the 21 functional members identified in humans only 13 have proteolytic capacity. Deregulation of ADAMs has been observed in several disease states, including cancer. ADAM9[88] has been observed to be upregulated in prostate, breast and intestinal cancer. Overexpression was associated with brain metastases in NSCLC and poor outcome in prostate

and renal cancer. In breast cancer ADAM9 even proved to be an independent predictor of response to tamoxifen.[89,90] With respect to pancreatic cancer, Grutzmann and coworkers identified ADAM9 as one of the genes upregulated in pancreatic ductal adenocarcinoma (PDAC) by gene expression profiling.[91] They expanded their research by evaluating the protein expression and prognostic significance of ADAM9 in PDAC and observed cytoplasmic ADAM9 to be correlated with poor tumour differentiation and identified ADAM9 as an independent predictor of poor survival in a set of 42 curatively (R0) resected PDACs (HR 2.85; $p < .05$).[92]

CX chemokine receptor 4 (CXCR4), originally identified as the cofactor for HIV to enter the cell,[93] is part of the chemokine receptor family. Chemokine receptors were initially found to play an important role in homing of leukocytes to sites of inflammation. Subsequently, they have been identified on a wide range of cells including cancer- and cancer stem cells. CXCR4 is the best studied receptor and selectively binds CXC chemokine stromal derived factor-1α (SDF-1α), also known as CXCL12. The SDF-1α-CXCR4 pathway seems to play a major role in cancer biology. High levels of SDF-1α in tissues and structures such as lymph nodes, liver, lung and bone are thought to direct metastases of CXCR4 expressing tumour cells.[94] In CXCR4 positive pancreatic adenocarcinoma cells, SDF-1α not only enhanced chemotaxis, transendothelial migration and matrigel invasion, it also stimulated cell proliferation and protected cells from serum deprivation-induced apoptosis.
Marechal and coworkers observed an association of high CXCR4 with lymph node metastases and recurrence in the liver and identified CXCR4 as an independent factor predicting poor survival in 71 patients that had undergone curative surgery for primary pancreatic adenocarcinoma ($p < .001$). Overall survival was 9.7 versus 43.2 months for high and low CXCR4 expression, respectively.[95]

DSC2 (desmocollin 2) is a member of the cadherin family, that constitutes one of three components of the desmosome, a complex of intercellular junctions that mediate cellular adhesion to promote tissue integrity and homeostasis. The other two components are the armadillo- and the plakin family. The desmosomal cadherin gene family constitutes of three desmocollins (Dsc 1-3) and four desmogleins (Dsg 1-4). Dscs and Dsgs are glycosylated type 1 transmembrane proteins, all encoded by different genes, and clustered at chromosome 18q12.1. They show tissue- and differentiation specific expression patterns.
Similarly, in some cancers upregulation of desmosome proteins was observed with associated enhanced tumour progression and reduced patient survival, while in others reduction of desmosome components was observed, also correlating with advanced grade, increased metastasis and poor prognosis. However, the majority of human cancer expression data and functional studies support a tumour suppressive role for desmosomes.[96, 97]
In pancreatic cancer, DSC2 seems to have a tumour suppressive function, correlating with lower tumour grade, clear lymph nodes and favourable outcome in a TMA of 115 microscopic radical resections (all $p < .029$).[98]

The epithelial cell adhesion and activating molecule (now unanimously called EpCAM, however also known by MK-1 and others) is a type I transmembrane glycoprotein, originally discovered as a cancer specific antigen on colorectal cancer.[99] EpCAM is expressed at the basolateral site of most epithelial cells and causes homophilic cell-cell adhesions, hypothetically preventing metastasis. However, EpCAM appears to have a dual role, abrogating E-cadherin mediated cell adhesion and inducing c-myc and cyclin A and E, which is illustrated by its controversial prognostic effects in different cancers.[100]
In pancreatic cancer EpCAM was identified as an independent prognostic factor predicting favourable outcome in a cohort of 95 patients following microscopic radical resection. 3-Year survival was 56.2 months for high EpCAM and 19.2 for low EpCAM expression (p= .0018). Previously it was already shown that this positive relation with survival is also true for resected carcinoma of the ampulla of Vater (p= .0097).[101] Akita and coworkers further showed that transfection of pancreatic cancer cells with EpCAM decreased migratory and invasive potential in vitro.[102]
Irrespective of its role, EpCAM expression appears to be epigenetically regulated, which opens possibilities for therapeutic interventions.

High mobility group A1 (HMGA1) proteins are nuclear DNA binding proteins that regulate transcription of a multitude of genes.[103,104] HMGA1 is increased during embryogenesis and becomes low or undetectable in adult tissue.[105] Neoplastic transformation has been associated with HMGA1 expression in several tumour types and an association with disease progression and poor survival was observed in several clinical studies. Besides invasion and metastases formation, HMGA1 is associated with growth and evading cell death.
Expression of HMGA1 in cancer cells and not in their normal counterparts makes it a particular interesting target for therapy. Our own group explored the expression of this protein in 99 pancreatic head and 112 periampullary tumours and identified HMGA1 as an independent predictor of poor outcome exclusively in periampullary cancer. Forty percent of patients lacking expression were alive 5 years following resection, compared to 22% of patients with HMGA1 expression (p= .017).[106]

Maspin (SERPINB5), a member of the Serine Protease Inhibitor family, was originally identified in 1994 as a candidate tumour suppressor gene in breast cancer.[107] The gene is located on chromosome 18 in close proximity to the frequently altered genes in pancreatic cancer, DCC and DPC4/SMAD4, and produces a 42kDa protein. Maspin is a multifunctional protein interacting with a wide variety of proteins, depending on its subcellular location, regulating cell adhesion, motility, apoptosis and angiogenesis.[108]
The expression of maspin is epigenetically regulated by methylation and acetylation, a process that seems tissue specific. While in breast cancer maspin was identified as a tumour suppressor, with hypermethylation of the promoter and

downregulation of protein expression, in contrast, in pancreatic cancer the promotor was shown to be hypomethylated and hyperacetylated with corresponding overexpression of the protein.[109] With respect to maspin as a prognostic marker in pancreatic cancer, Cao and colleagues identified Maspin as an independent factor predicting poor outcome in 223 surgically resected pancreatic cancer specimens (HR 2.433; p= .0106). Furthermore, they showed maspin expression in both low- and high grade PanINs, suggesting that epigenetic regulation is an early process in the development of pancreatic cancer.[110]
Additionally, Lim and coworkers also identified maspin expression as an unfavourable prognostic factor in a cohort of 72 patients with pancreatic ductal adenocarcinoma (p= .022). However, this cohort consisted of 25 stage IV cancers, nonetheless resection was successful.[111]
The lack of expression in normal pancreatic cells makes maspin an interesting target in for instance neo-adjuvant therapy.

A group of proteins thoroughly investigated in association with cancer by a Japanese group are the mucins. Mucins are high molecular weight glycoproteins secreted by epithelial cells to protect and lubricate the epithelial cell surface. However they have also demonstrated to play a role in signal transduction and oncogenesis. There are two subfamilies of mucins, the secreted and the membrane bound. Yonezawa and colleagues demonstrated that the membrane bound MUC1 and MUC4 are related to aggressive behaviour and poor outcome, whereas the secretory MUC2 is associated with indolent behaviour of human neoplasms.[112]
It was this latter one that was associated with improved survival in a cohort of 59 curatively resected cases of pancreatic ductal adenocarcinoma. Median survival in patients with positive MUC2 expression was 44 months, versus 16 months in patients with negative MUC2 expression (p= .003).[113] Under physiological circumstances MUC2 is expressed in a limited ammount of tissues such as intestine, bronchus and salivary gland.
Another mucin identified as a prognostic marker in pancreatic cancer was the membrane bound MUC17. Hirono and coworkers observed high MUC17 to be independently associated with lymph node metastasis and poor survival in a set of 63 patients that received radical resection of PDAC (p= .0192 and .0001 for lymph node metastasis and survival, respectively)[38] MUC 17 is mainly expressed in the digestive tract, including the duodenum, ileum and colon. Apart from the membrane bound form, it also has a soluble form that is generated by alternate splicing. MUC17 was found to be overexpressed in pancreatic cancer compared to normal pancreas or pancreatitis. Although the function of MUC17 is still unknown, its similarity to MUC1 and its EGF-like domain, suggest a role in growth, differentiation and immunoregulation. Both MUC2 and MUC17 expression seem to be epigenetically regulated by DNA methylation and histone modification.[114]

Pepsinogen C or progastricsin (PGC) is the precursor of gastricsin, a gastric proteinase belonging to a family of aspartyl proteinases. It was first purified and isolated from human gastric juice in 1959.[115] The progastricsin gene is located on

chromosome 6. Although gene expression depends on various factors, hormonal regulation seems to play an important role. Of the five pepsinogens described, pepsinogen A and C are mainly expressed in adult vertebrates, whereas prochymosin and pepsinogen F are specific for fetal/infant stages. Tissue expression of most pepsinogens is primarily restricted to the stomach, however pepsinogen C is also expressed in other tissues, amongst which the pancreas, but also in several cancers. In an acidic environment pepsinogen C is activated by stepwise cleavage. The proteolytic activity of pepsinogen C plays an important role in many biological processes.[116]
With respect to pancreatic cancer, PGC was associated with more differentiated tumours and identified as a favourable prognostic marker following curative resection in a cohort of 67 adenocarcinomas. Median survival was 30 and 12 months for patients with and without PGC expression, respectively ($p > .05$).[117]

In addition, we recently published the first natural barrier to tumour invasion and metastases, the basement membrane (BM), in particular the presence of one of its major components, laminin, to be of prognostic value in curatively resected pancreatic head cancer. More than twice as many patients were recurrence free and alive 5-years following resection if their tumours showed ≥25% epithelial BM lining by laminin as compared to tumours showing limited BM lining ($p = .034$).[118]
Basement membranes are not merely static structures; they are continuously being degraded, synthesized and remodelled. Under physiologic conditions a coordinated equilibrium exists between basement membrane synthesis and breakdown. However in cancer basement membrane deposition and breakdown is disorganized, resulting in fragmented basement membranes.[119-121] These fragmented basement membranes have been observed in pancreatic cancer by us and several other groups.[122-125]

Discussion

Although data unravelling the genetic alterations accompanying the progression from normal pancreatic epithelium through the different PanIN stages into pancreatic cancer have increased,[126] and many additional genes and proteins have been evaluated as prognostic markers in pancreatic cancer,[12-16] patient tailored treatment as with oestrogen/progesterone receptor and HER2NEU status in breast cancer is still beyond our reach. Generally studies evaluate heterogeneous patient sets, negatively influencing interpretational value. We therefore decided to search for a more valuable prognostic signature by selecting markers identified exclusively in curative (R0) resections.
Following a systematic review of all English literature potentially fulfilling our inclusion criteria, 18 prognostic markers were identified and grouped according to the recently updated hallmarks of cancer by Hanahan.[17] Cancer growth is facilitated by uncontrolled proliferative signalling and evading growth suppression, the two first hallmarks as described by Hanahan and coworkers. Three markers control-

ling proliferation were identified as independent prognostic makers. Activated Akt, HDGF and Ki67, all affected pancreatic cancer prognosis negatively.[22,26,29] Additionally, the recently published rate limiting enzyme in the novel synthesis of DNA, TS, was discussed. In contrast to the other three proteins, TS predicted favourable outcome following curative resection.[32]

With respect to growth suppression, two markers were identified; AP-2α and PML.[38,43] The well known p53 and TGFβ, were also discussed in this context.

Under physiological circumstances cells respond to damage induced by stress signals with apoptosis. Cancer cells however, generally circumvent this mechanism. Three proteins involved in apoptosis were found to be of value in predicting pancreatic cancer prognosis; Bax and the recently published Bcl-2 associated protein Bag-1 were associated with favourable outcome, whereas VCP expression predicted poor outcome.[52,62,70]

Another tumour feature, generally accepted as of paramount importance for tumour growth and metastasis formation, is angiogenesis. Pancreatic cancer however is known for its extensive extracellular matrix deposition, called desmoplasia, creating increased intratumoural pressure and tumour-vessel distances, resulting in a hypoxic environment.[127-129] Two small studies identified angiogenesis as an independent marker predicting poor outcome following curative resection of pancreatic cancer.[75,77] Whereas, our own group failed to identify a relation with prognosis in a much larger patient set.[80] The prognostic value of angiogenesis as a prognostic marker in pancreatic cancer therefore remains controversial.

In order to spread to distant sites a tumour needs to invade and metastasize. In line with observations in other cancers, the first natural barrier to this process, the basement membrane, proved of value in determining prognosis following resection. Interrupted basement membrane delineation by laminin was associated with poor outcome following curative resection of pancreatic head cancer. Nine additional proteins involved in invasion and metastases formation were found to be of prognostic value. ADAM9, CXCR4, maspin, MUC17 and pepsinogen C predicted poor outcome following curative resection of pancreatic cancer,[38,92,95,110] an effect observed in periampullary cancer for HMGA1,[106] whereas DSC2, EpCAM, MUC2 and PSC proved to have a tumour suppressive function.[98,101,102,113,130]

Most of the above mentioned changes regulating cell proliferation, survival and dissemination require genetic alterations, which can be acquired by either mutation or epigenetic changes. Epigenetic refers to a heritable change in the pattern of gene expression that is mediated by mechanisms other than alterations in the primary nucleotide sequence of a gene. The difference between mutations and epigenetic changes is that the latter is reversible. Examples of epigenetic changes are DNA methylation and histone modification. DNA is wrapped around an octamer of histones to form the nucleosome. DNA methylation creates tightly packed and regularly spaced nucleosomes, unfavourable for protein access to stimulate gene transcription. Under physiological circumstances gene promoters are usually unmethylated, whereas in most cancers these promoter regions are methylated. This methylation in promoter regions causes aberrant repression of gene transcription, which is a mechanism to silence tumour suppressor genes.[131]

Although we did not identify any specific epigenetic modifications to be of prognostic value following resection of pancreatic or periampullary cancer, many of the identified markers are in fact epigenetically regulated.
Unfortunately, except for the tumour micro-environmental factor angiogenesis and the proliferation marker Ki-67, we were only able to identify a single study for each of the 21 markers fulfilling our selection criteria. And for angiogenesis, conclusions were not even unanimous. A large amount of studies was excluded because of inclusion of either advanced disease or residual tumour following resection. We experienced however that reporting on resection margin is often poor. And as Verbeke and coworkers recently illustrated, standardisation with respect to evaluating resection margins, but also the origin of the tumour (i.e. pancreatic or periampullary), is important for accurate prognostication.[132] Additionally, with a few exceptions, most makers identified, were analysed in relatively small patient sets. We therefore have to conclude that although we have identified a set of interesting prognostic markers, they do need confirmation by others, in larger patient sets.
Generally, metastases determine pancreatic cancer prognosis and one could argue that measuring primary tumour characteristics is therefore not representative of prognosis, especially in case of complete microscopic resection of the primary tumour. Indeed primary tumour protein expression is not always reflective of the expression levels in their metastatic counterparts.[132] In fact, during malignant progression microenvironmental pressure initiates phenotypical changes enabling cells to overcome stressful circumstances and metastasize. Similarly at metastatic sites, different microenvironmental circumstances, could give rise to different phenotypes.[133] However, it is these primary tumour characteristics that enable the tumour to metastasize in the first place. Recently this trait was assigned to so-called cancer stem cells.[134] A subset of cells also identified in pancreatic cancer,[135] that need further evaluation.
The progression of pancreatic cancer and the formation of metastases is a multi-step process regulated by the dynamic interaction of tumour cells with the surrounding tumour stroma. Although we aimed at identifying both as prognostic markers in pancreatic cancer, apart from basement membrane component laminin, no stromal proteins were identified in the current review. Our own group is currently investigating the prognostic value of two important extracellular matrix components, collagen type I and fibronectin.
None of the studies described, evaluate response to adjuvant treatment. Marechal and coworkers, however recently found a striking survival difference between high and low hENT1 expression in tumours of 45 patients following R0 resection of pancreatic head cancer that received adjuvant treatment with gemcitabine. 3-Year survival rates were 68.4% and 19.2% for high and low expressors, respectively (p= .0007). For hCNT3 and dCK survival rates were twice as high for high expressors compared to low expressors (p= .003 and .03 for hCNT3 and dCK respectively).[136,137] Although gemcitabine is currently not the standard treatment following curative resection of pancreatic cancer, these markers could differentiate between patients that would benefit from adjuvant treatment with gemcitabine and those that would not.

In conclusion, although studies confirming the prognostic value of the markers described, are needed, the current systematic review was able to identify a subset of proteins that could be of value in identifying certain prognostic subgroups following curative resection of pancreatic and periampullary cancer, and consequently aid in therapeutic decision making.

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Summary / Samenvatting

Summary

The limitations of current treatment protocols for pancreatic cancer warrant an alternative approach. Experts claim treatment should be individualised to the patient guided by prognostic and predictive markers.
The search for potential biomarkers to optimize and improve treatment of patients with pancreatic cancer starts with a better understanding of the biology of pancreatic cancer. In chapter 2, a brief overview is given of the current understanding of the molecular biology of pancreatic ductal adenocarcinoma, including the progression model for pancreatic cancer with its accompanying genetic alterations. Furthermore, growth factors and their receptors overexpressed in pancreatic cancer and several signalling cascades relevant for the behaviour of pancreatic cancer were described. Techniques to identify genetic alterations and models to study the biology of pancreatic cancer were also outlined. To conclude some inherited genetic alterations are described.
Previously pancreatic cancer research focused mainly on the tumour cell characteristics. Lately however, focus is shifting towards a more tumour stroma based approach. Tumour stroma is a compartment present in excessive amounts in pancreatic cancer and one that seems to be actively involved in tumour formation, progression, invasion and metastasis. We therefore investigated both tumour cell and stromal features as prognostic factors in pancreatic head and periampullary-cancer. Since survival of patients with incomplete resections is no different from that of patients with locally advanced, surgically unresectable tumours treated with chemoradiation, exclusively microscopic radical resections were used for our prognostic studies. Furthermore, in order to identify its subcellular location, we investigated protein expression by immunohistochemistry.
In chapter 3 the expression and prognostic significance of major target of 5-FU, and rate limiting enzyme in the novel synthesis of DNA, thymidylate synthase (TS), was investigated in 212 resected pancreatic head and periampullary cancers. Two anatomically closely related tumours in which a different effect was observed following 5-FU based adjuvant chemoradiotherapy (CRT) in the EORTC trial. Only a small proportion of pancreatic head and periampullary cancers showed high TS expression. It was this group of patients that experienced a relatively favourable outcome following curative resection, compared with the ones lacking or showing low levels of TS. However, this was only true for patients diagnosed with pancreatic head and not periampullary cancer. This suggests that these tumours are different biological entities with respect to TS and might therefore also be differently affected by adjuvant treatment with 5-FU based CRT.
Another feature of cancer is evasion of cell death or apoptosis. Pancreatic cancer cells are type II cells in which mitochondria are required as signal amplifiers to activate executioner caspases and induce apoptosis. Bcl-2 plays a major role in this mitochondrial driven death pathway. In the search for novel interaction partners, Bcl-2 associated anthanogen-1 (Bag-1) was identified. In chapter 4 Bag-1 expression and its prognostic value were explored. Both nuclear and cytosolic expression were observed, however cytosolic expression was almost exclusively associated with nuclear expression. With respect to prognosis, nuclear Bag-1

proved to be an independent factor predicting favourable outcome following curative resection of pancreatic head cancer. In fact, patients lacking expression had such poor survival that the benefit of resection could be questioned.
The formation of metastases is a multi-step process guided by the interaction of the tumour with its environment. For a tumour to grow beyond a certain size and in order to metastasize, tumours need angiogenesis. Consequently the amount of tumour microvessels has been associated with tumour recurrence and poor outcome in several solid tumours. However, not all tumours or tumour subtypes seem equally dependant on angiogenesis. In chapter 5 microvessel density (MVD) was measured using the pan-endothelial marker CD31 and quantified using an automated system in both pancreatic head and periampullary cancer. Periampullary cancers appeared to be more vascularised than pancreatic head cancers and although angiogenesis correlated with nodal involvement in pancreatic head cancer and thus gives some information on the malignant potential, our data suggest that pancreatic cancer is an example of a tumour in which prognosis is less dependent on angiogenesis.
However before a tumour cell can even reach a tumour vessel it needs to cross several barriers of which the basement membrane (BM) constitutes the first. Basement membranes are not just static structures; they are continuously being remodelled by glycoprotein rupture and synthesis. Conceivably, the presence of basement membrane is the net effect of tumour stroma interaction and consequently a reflection of tumour behaviour. Widely fragmented BMs have been associated with adverse outcome in several cancers. In chapter 6 basement membrane continuity quantified by its major components, laminin and collagen type IV, was studied with respect to its relation with pancreatic cancer outcome. Most tumours showed widely fragmented BMs. Delineation of both BM components was weakly correlated with each other; nonetheless it was only BM delineation by laminin that correlated with outcome of pancreatic head cancer.
Another protein involved in invasion and metastases, high mobility group 1 (HMGA1), was discussed in chapter 7. HMGA1 is an architectural transcription factor that regulates transcription of a multitude of genes by interacting with the transcription machinery and altering chromatin structure. HMGA1 was expressed in 47% and 26% of pancreatic head- and periampullary cancers respectively and predicted an adverse outcome following curative resection of periampullary cancer. 5-year survival was 25% and 44% for patients with tumours showing or lacking HMGA1 respectively. The lack of expression in normal tissues makes HMGA1 a particularly interesting target for therapy.
To conclude, in chapter 8 a systematic review is given describing all independent prognostic factors for curatively resected pancreatic head and periampullary cancer. Although most prognostic biomarker studies are performed on heterogeneous patient groups, including both localized and advanced disease, we were able to identify 17 additional markers predicting outcome following curative resection of pancreatic cancer. Nonetheless, these markers need validation in larger patient sets before they can be implicated in clinical practice.

Samenvatting

De beperkingen van de huidige behandeling van het pancreas carcinoom vragen om een alternatieve aanpak. Experts zijn van mening dat de behandeling geïndividualiseerd dient te worden naar de patiënt, aan de hand van prognostische en predictieve tumormarkers.

De zoektocht naar geschikte biomarkers om de behandeling van pancreas carcinoom te optimaliseren, vereist een beter begrip van het onstaan van het pancreas carcinoom. In hoofdstuk 2 wordt een kort overzicht gegeven van de huidige kennis ten aanzien van de ontstaanswijze van het pancreas carcinoom, waarbij het progressiemodel van het pancreas carcinoom wordt besproken inclusief de begeleidende genetische veranderingen. Verder worden groeifactoren die tot overexpressie komen in het pancreas carcinoom, evenals hun receptoren en verschillende signaal transductie pathways, die van belang zijn voor het gedrag van het pancreas carcinoom, besproken. Technieken om genetische veranderingen te indentificeren en diermodellen voor het bestuderen van de ontwikkeling van het pancreascarcinoom worden eveneens beschreven. Tot slot worden enkele erfelijk bepaalde genetische veranderingen besproken.

Voorheen richtte onderzoek naar het pancreascarcinoom zich met name op de karakteristieken van de tumor zelf, tegenwoordig is er ook aandacht voor tumor stroma karakteristieken. Tumor stroma is een compartiment dat in overvloed aanwezig is in het pancreascarcinoom en dat van belang lijkt voor de ontwikkeling en de progressie van het pancreas carcinoom. Derhalve is gekozen om zowel naar tumor- als stroma karakteristieken te kijken in de zoektocht naar prognostische markers voor pancreaskop en periampullair carcinoom. Aangezien de overleving van patiënten met irradicale resecties niet anders is dan van lokaal gevorderd, niet-resectabel pancreas carcinoom, behandeld met chemo-radiotherapie, hebben we ons voor onze prognostische studies beperkt tot microscopisch radicaal gereseceerde tumoren. Om tevens de lokatie van expressie te kunnen analyseren, hebben we immunohistochemie gebruikt voor de bepaling van de expressie van de verschillende potentieel prognostische factoren.

Hoofdstuk 3 beschrijft de expressie en prognostische waarde van de primaire target van 5 FU, betrokken bij de novo synthese van DNA, TS, in 212 gereseceerde pancreaskop en periampullaire tumoren; twee anatomisch nauw gerelateerde tumoren met een verschillend effect op radiochemotherapie met 5-FU in de EORTC trial. Slechts een klein percentage van de pancreas kop en periampullaire tumoren bracht TS in sterke mate tot expressie. Het waren echter wel deze tumoren die een relatief gunstige prognose lieten zien, volgend op resectie ten opzichte van de tumoren met een lage tot afwezige expressie. Dit was alleen van toepassing op patiënten met pancreaskop carcinoom en niet op de patiënten met periampullaire tumoren. Dit suggereert dat beide verschillende tumoren zijn voor wat betreft TS en daarmee mogelijk ook verschillend beïnvloed worden door 5-FU gebaseerde chemoradiotherapie.

Een andere eigenschap van kanker is het ontsnappen aan geprogrammeerde celdood (i.e. apoptose). Pancreas kanker cellen zijn type II cellen die mitochondria nodig hebben als katalysatoren van de caspases voor het induceren van apop-

tose. Bcl-2 speelt een prominente rol in deze mitochondriale apoptose pathway. In de zoektocht naar nieuwe interactie partners van Bcl-2, is Bag-1 geïdentificeerd. In hoofdstuk 4 worden de expressie en de prognostische waarde van Bag-1 besproken. Expressie werd zowel in het cytoplasma als in de nucleus waargenomen, waarbij expressie in het cytoplasma in vrijwel alle gevallen gepaard ging met nucleaire expressie. Bag-1 bleek een gunstige prognostische factor voor patiënten waarbij resectie van een pancreaskop tumor had plaatsgevonden. De prognose van patiënten waarvan de tumoren Bag-1 niet tot expressie brachten, was dusdanig slecht, dat men zich moet afvragen of resectie voor die patiëntengroep wel van toegevoegde waarde is.
Het uitzaaien van tumoren naar andere lokaties (metastasering) is een proces dat opgebouwd is uit verschillende stappen, die afhankelijk zijn van de interactie van de tumor met zijn omgeving. Boven een bepaalde grootte en om uit te kunnen zaaien, hebben tumoren bloedvaten nodig. De hoeveelheid aanwezige bloedvaten is in verscheidene solide tumoren dan ook geassocieerd met tumor recidieven (terugkeer van de ziekte) en metastasen. Echter niet alle tumoren of tumor subtypes lijken in dezelfde mate afhankelijk van de vaatvoorziening. Hoofdstuk 5 beschrijft een analyse van de vaatdichtheid, waarbij de expressie van de multi-endotheel marker CD31 is gekwantificeerd middels een automatisch systeem in zowel kop als periampullaire tumoren. Periampullaire tumoren bleken meer gevasculariseerd dan kop tumoren, en ondanks het feit dat de vaatdichtheid gecorreleerd was aan lymfekliermetastasen in pancreaskop carcinoom en daarmee enige informatie geeft over de maligne potentie, suggereren onze data dat het pancreas carcinoom een voorbeeld is van een tumor die minder afhankelijk is van de vaatvoorziening dan sommige andere tumoren.
Echter voordat een tumorcel überhaupt bij een bloedvat kan komen, moet deze enkele barrieres doorbreken, waarvan de basaal membraan de eerste is. Basaal membranen zijn geen statische structuren, maar worden continu geremodelleerd door de aanmaak en afbraak van eiwitten. Het is dan ook niet onvoorstelbaar dat de continuiteit van de basaal membraan een reflectie is van tumor-stroma interactie en daarmee van het gedrag van de tumor. Uitgebreid onderbroken basaal membranen zijn geassocieerd met een slechte prognose in verscheidene tumoren. Hoofdstuk 6 beschrijft de continuiteit van de basaal membraan, gekwantificeerd aan de hand van zijn primaire componenten laminine en collageen type IV en de correlatie met de prognose van pancreas carcinoom. De meeste tumoren toonden een sterk onderbroken basaal membraan. De expressie van beide componenten correleerde met elkaar, echter alleen de expressie van laminine was van belang voor de prognose van pancreaskop tumoren.
Een ander eiwit betrokken bij het proces van tumor invasie en metastasering, is HMGA1, welke wordt besproken in hoofdstuk 7. HMGA1 is een transcriptiefactor welke door interactie met het transcriptiecomplex, en daarmee verandering van de chromatine structuur, de transcriptie van een veelheid aan genen reguleert. HMGA1 werd door respectievelijk 47% van de pancreaskop en 26% van de periampullaire tumoren tot expressie gebracht en voorspelde een slechte overleving na curatieve resectie van periampullaire tumoren. De 5-jaars overleving bedroeg

25% voor patienten met tumoren die HMGA1 tot expressie brachten en 44% voor patiënten met tumoren die dit eiwit niet tot expressie brachten. De afwezigheid van HMGA1 in normaal weefsel maakt het een interessant eiwit voor therapeutische targeting.
Tot slot, beschrijft hoofdstuk 8 een systematische review van alle prognostische markers voor gereseceerde pancreas en periampullaire tumoren. Ondanks het feit dat de meeste prognostische studies heterogene groepen beschrijven met zowel lokale als uitgebreide ziekte, waren we in staat 17 prognostische markers te indentificeren. Deze markers dienen echter wel gevalideerd te worden in grotere studies voordat ze geïmplementeerd kunnen worden in de dagelijkse praktijk.

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List of Publications

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- Van der Zee JA, Van Eijck CHJ, Ten Hagen TLM. Prognostic markers following curative resection of pancreatic cancer: a systematic review. Submitted.

Presentaties

- Van der Zee JA, Tran TCK, Tilanus HW. The value of routine contrast radiology in diagnosing anastomotic leaks following oesophagectomy for oesophageal cancer. Meeting of the International Hepato-Pancreato-Biliary Association (IHPBA, Edinburgh, Sept 2006).

Posters

- Van der Zee JA, Ten Hagen TLM, Hop WCJ, Dicheva BM, Seynhaeve ALB, Koning GA, Eggermont AMM, Van Eijck CHJ. Bcl-2 associated anthanogen-1 (Bag-1): a novel prognostic marker in adenocarcinoma of the pancreatic head. Joint mee-ting of European Cancer Organisation (ECCO) and European Society for Medical Oncology (ESMO) (Berlijn, Sept 2009).
- Van der Zee JA, Poley J, Hermans JJ, Van Eijck CHJ. The value of endoscopic ultrasound in the workup of focal lesions of the pancreas. Meeting of the International Hepato-Pancreato-Biliary Association (IHPBA) (Edinburgh, Sept 2006).

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1) Voorjaarsvergadering NVVH (2005);
2) Meeting of the International Society of Diseases of the Esophagus (Adelaide, Feb 2006).
- Van der Zee JA, Van der Hoeven JAB, Struik K, Eeftinck Schattenkerk M, Eddes EH. Slokdarmresecties, de behandeling van naadlekkages na een thoracale anastomose. Najaarsvergadering NVVH (2004).

Curriculum vitae

Jill Annelien van der Zee, was born on June 25th 1980, in Groningen, the Netherlands. She attended secondary school at “Montessori Lyceum Herman Jordan” in Zeist and graduated in 1998.
Subsequently she started Medical School at the University of Groningen. In 2001 she completed the subjects “Introduction to law” and “Medical law” at the Faculty of Law. In 2002 she went to Adelaide in Australia for her scientific internship, where she was involved in a project on pi-GST as a screening method for urinary tract infections (Dr. P. Henning). During her stay she also performed a systematic review comparing laparoscopic versus open pyloromyotomy (Prof. Dr. H.L. Tan). She completed her general rotations in Deventer Hospital and decided to do her final rotations at the department of Surgery at Erasmus MC in Rotterdam. As a student, she was involved in several research projects.
Following her graduation from Medical School in 2005 Jill started her PhD at the Department of Surgery at Erasmus MC. During her PhD, she spent one and a half years at Brigham and Women’s Hospital/Harvard Medical School in Boston, where the foundation was laid for some of the work presented in this thesis.
In 2010 Jill worked as an intern at the Department of Surgery at Erasmus MC, which was followed by an appointment at the Department of Surgery at MC Haaglanden in The Hague in 2011. She is currently working as an intern at de Department of Urology at Westfriesgasthuis in Hoorn.