PROGNOSTIC TUMOUR MARKERS IN PANCREATIC CANCER

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Academic Dissertation

To be presented for public examination with the permission of
the Medical Faculty of the University of Helsinki,
in the Auditorium 2,
Meilahti Hospital, Haartmaninkatu 4,
on February 3rd, 2006, at 12 noon.

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ISBN 952-91-9894-9 (paperback)

ISBN 952-10-2935-8 (PDF)

Yliopistopaino

Helsinki 2006

To my family

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-V.

I Juuti Anne, Nordling Stig, Louhimo Johanna, Lundin Johan, von Boguslawski Kristina, Caj Haglund:

Loss of p27 expression is associated with poor prognosis in stage I-II pancreatic cancer.

Oncology 65; 371-377: 2003.

II Juuti Anne, Nordling Stig, Lundin Johan, Louhimo Johanna, Haglund Caj:

Syndecan-1 expression – a novel prognostic marker in pancreatic cancer.

Oncology 68; 97-106: 2005.

III Juuti Anne, Nordling Stig, Louhimo Johanna, Lundin Johan, Haglund Caj:

Tenascin C expression is upregulated in pancreatic cancer and correlates with differentiation.

Journal of Clinical Pathology 57; 1151-1155: 2004.

IV Juuti Anne, Louhimo Johanna, Nordling Stig, Ristimäki Ari and Haglund Caj: Cyclooxygenase-2 expression correlates with poor prognosis in pancreatic cancer.

Journal of Clinical Pathology, in press.

V Juuti Anne, Lundin Johan, Nordling Stig, Louhimo Johanna, Haglund Caj:

Epithelial MMP-2 expression correlates with worse prognosis in pancreatic cancer.

In manuscript.

2. ABBREVIATIONS

ABC	avidin-biotin complex
BRCA	breast cancer gene
CA	carbohydrate antigen
CDKI	cyclin-dependent kinase inhibitor
CEA	carcinoembryonic antigen
CI	confidence interval
COX	cyclooxygenase
CT	computed tomography
DNA	deoxyribonucleic acid
DPC4	deletet in pancreatic cacner, locus 4, also called SMAD4 and MADH4
ECM	extracellular matrix
EGF	epidermal growth factor
erbB2	erythroblastic leukemia oncogene homolog 2
ERCP	endoscopic retrograde cholangiopancreaticography
EUS	endoscopic ultrasonography
FGFR1	fibroblast growth factor type I receptor
GAG	glycosaminoglycan
HER-2	human epidermal growth factor receptor 2
HR	hazard ratio
HSPG	heparane-sulphate proteoglycan
Mab	mouse monoclonal antibody
MMP	matrix metalloproteinase
MRCP	magnetic resonance cholangiopancreaticography
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MUC	mucin
OR	odds ratio
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate buffered saline
PET	positrion emission tomography
TF	tissue factor
TGF	transforming growh factor
TNM	tumour node metastasis
TP53	tumour protein p53
UICC	Union Internationale Contre le Cancer
US	ultrasonography
WHO	World Health Organization

3. ABSTRACT

Background. Pancreatic cancer is one of the major causes of cancer death in the industrialised world. The overall survival of patients with ductal pancreatic adenocarcinoma is poor: 5-year survival is only 0.2 to 4%. Tumour stage and histological grade are used as prognostic markers in pancreatic cancer. However, there are differences in survival within stages and histological grades. New, additional and more accurate prognostic tools are needed.

Aims. The purpose of this study was to investigate whether the tissue expression of potential and promising tumour markers p27, tenascin C, syndecan-1, COX-2 and MMP-2 are associated with clinicopathological parameters in pancreatic cancer. The expression of p27, tenascin C and syndecan-1 was also evaluated in acute and chronic pancreatitis. The main purpose in the study was to find new prognostic markers for pancreatic adenocarcinoma.

Patients. The study included 147 patients with histologically verified pancreatic adenocarcinoma treated at Helsinki University Central Hospital from 1974 to1998.

Methods. The expression of tumour marker antigens was demonstrated by immunohistochemistry using monoclonal antibodies against p27, syndecan-1, tenascin C, COX-2 and MMP-2. The results were compared with clinicopathological variables, i.e. age, sex, TNM stage and histological grade. Survival analyses were performed with univariate Kaplan-Meier life-tables and the log-rank test, while multivariate analyses were performed using Cox regression.

Results. Pancreatic adenocarcinomas expressed p27, syndecan-1, tenascin C, COX-2 and MMP-2 in 30, 94, 92, 36 and 50% of the samples, respectively. Loss of p27 expression was associated with poor prognosis in stage I and II pancreatic cancer. Stromal syndecan-1 expression was an independent prognostic marker in pancreatic cancer, whereas epithelial syndecan-1 expression predicted better prognosis only in stage I and II disease. Tenascin C expression did not correlate with survival but was associated with differentiation. COX-2 expression was associated with poor outcome and was an independent prognostic factor. Epithelial MMP-2 correlated with poor prognosis in pancreatic cancer.

Conclusion: p27 and epithelial syndecan-1 are prognostic markers in early (stage I and II) pancreatic cancer. Stromal syndecan-1, COX-2 and epithelial MMP-2 are prognostic factors in ductal pancreatic adenocarcinoma.

4. INTRODUCTION

Pancreatic cancer is the third most prevalent of gastrointestinal tract malignancies, outnumbered only by colorectal and gastric cancer. The incidence of pancreatic cancer in Finland for 2003 was 8.5/100,000 for males and 7.3/100,000 for females. The incidence has been rising steadily throughout the 1970´s and 1980´s, and reaching a steady state by 1990 (Fernandez et al. 1994). According to the Finnish Cancer Registry in 2003 pancreatic cancer was the third leading cause of cancer death after lung and prostate cancer in men, and breast and lung cancer in women.

Pancreatic cancer metastasizes to lymph nodes and distant organs early in the course of the disease. Due to the anatomic location, small tumours in the pancreatic head readily invade the duodenum, common bile duct and great vessels. Thus, at the time of first symptoms, pancreatic tumours have often infiltrated surrounding organs rendering surgical intervention impractical. The biological nature of the disease is still unclear: little is known about the aetiology and why it is so aggressive. Moreover, the aetiology of pancreatic cancer seems to be multifactorial. Changes in the incidence of pancreatic cancer could be explained by lifestyle related factors. Smoking and chronic pancreatitis are considered as risk factors for pancreatic cancer (Silverman et al. 1994; Boyle et al. 1996; DiMagno et al. 1999; Talamini et al. 1999; Li et al. 2004).

Histological type, histological differentiation and stage of disease are the gold standard factors for assessing the prognosis of patients with pancreatic cancer (Roder 2001). However, there are differences in survival within these subgroups. New and better prognostic indicators are needed. The aim of this study was to assess the prognostic impact of a series of promising biologic factors.

5. REVIEW OF THE LITERATURE

EPIDEMIOLOGY

Incidence and Mortality

Approximately 170 000 new cases of pancreatic cancer are diagnosed world-wide yearly, with an incidence of 5 to 10 per 100 000 in European countries (Fernandez et al. 1994; Parkin et al. 1999). The incidence has been rising in most European countries from the 1950´s to the 1980´s, after which there has been a plateau in the 1990´s (Fernandez et al. 1994). World-wide, industrialised countries have a higher incidence than developing countries: In Japan the incidence is 9.0-10.9 for men and 4.7- 5.7 for women, while in the USA the incidence is 11.8 for black men and 7.7 for white men. The lowest rates are found in Africa and some Asian countries (<2/100 000) (Parkin et al. 1997).

In Finland, the age adjusted incidence in 2003 was 8.5 per 100 000 person-years for males and 7.3 per 100 000 for females (Table 1). Pancreatic cancer constitutes approximately 3% of all diagnosed cancers. In 2003 there were 368 males and 476 females that were diagnosed with pancreatic cancer.

At the national level, the geographic incidence for men is highest in Länsi-Pohja (10.8) and Kymenlaakso (10.8), while the lowest incidence is in Pohjois-Savo (6.4) and Satakunta (6.9).

Table 1. The incidence and mortality of pancreatic cancer by period according to Finnish cancer registry. Updated 14.10.2005

Period of time	1957-1961	1962-1966	1967-1971	1972-1976	1977-1981	1982-1986	1987-1991	1992-1996	1997-2001	2002	2003
No. of new cases											
Male	146	170	224	255	275	303	311	324	363	366	**368**
Female	120	157	195	256	293	349	393	400	417	441	**476**
Rates*											
Male	7.6	8.2	9.7	10.2	10.0	10.2	9.6	9.0	9.1	8.5	**8.5**
Female	4.3	5.1	5.5	6.4	6.4	6.8	7.0	6.6	6.5	6.7	**7.3**
Mortality per period											
Male	139	160	214	236	260	280	299	298	338	346	**357**
Female	113	153	184	242	277	327	376	375	390	417	**487**

*Age-adjusted incidence rates of cancer per 100,000 person-years

Mortality almost equals incidence since there are very few survivors of pancreatic adenocarcinoma (Alanen et al. 1993; Carpelan-Holmström et al. 2005). In Finland, in 2003 there were 844 pancreatic cancer deaths and an equal amount of new pancreatic cancers (www.cancerregistry.fi).

Risk factors and molecular epidemiology

The lifetime risk of pancreatic cancer is approximately 1% in developed countries (Parkin et al. 1999). Risk factors consistently reported are older age and cigarette smoking, although family history, diabetes and chronic pancreatitis are also associated with a higher risk of pancreatic cancer. In some studies alcohol consumption, obesity and a western diet have been proposed as additional risk factors (Li et al. 2004).

Pancreatic cancer is a disease of the elderly. Over 80% of cases are from patients between the ages of 60 and 80, with a mean of 66 years (Gold et al. 1998). Pancreatic cancer almost never occurs before the age of 20 and is extremely rare before 40. According to the Finnish cancer register only two patients under the age of 40 had pancreatic cancer in 2002. However, in familial pancreatic cancer the mean age at diagnosis is about 20 years lower than in sporadic pancreatic cancer (James et al. 2004). Early onset of pancreatic cancer has also been associated with hereditary pancreatitis, BRCA2 gene mutations and cigarette smoking (Lal et al. 2000; Rulyak et al. 2003; Luttges et al. 2004). Ethnic origin has also been reported as a risk factor: The highest incidence of pancreatic cancer in men is reported in New Zealand Maoris, native Hawaiians and black Americans (Boyle et al. 1989).

Epidemiological studies have confirmed that relatives of pancreatic cancer patients have an increased risk, which is 50-fold higher than patients with no family history of pancreatic cancer (Fernandez et al. 1994; Lowenfels et al. 2001). Growing evidence suggests the presence of a single autosomal dominant gene in familial pancreatic cancer kindreds, and a susceptibility locus on chromosome 4 has recently been identified in one such family (Rulyak et al. 2004). There are also several germ line mutations associated with pancreatic cancer (Vaidya et al. 1996; Neoptolemos et al. 1997; Su et al. 1999). A inherited germ line disorder is BRCA2 mutation, which is found in 7-10% of sporadic pancreatic cancer patients and in 15-20% of patients with a strong family history of pancreatic cancer (Lal et al. 2000; Keleg et al. 2003). Several genetic syndromes are associated with an increased risk of pancreatic cancer, such as hereditary pancreatitis (70- to 100-fold risk), hereditary non-polypous colorectal cancer, Peutz-Jegher syndrome (up to 132-fold risk), familial breast cancer (3.5-to 10-fold risk) and familial atypical multiple melanoma (13-to 65- fold risk) (Hruban et al. 1998). These inherited genetic disorders are thought to cause about 10% of all pancreatic cancers.

Chronic, hereditary and tropical pancreatitis are risk factors for pancreatic cancer (Lowenfels et al. 1993; Chari et al. 1994; Bansal et al. 1995; Lowenfels et al. 1997). However, acute pancreatitis, history of alcohol consumption or gallstones are not associated with pancreatic cancer (Bansal et al. 1995). The highest risk is seen in hereditary pancreatitis in which the cumulative risk for pancreatic cancer is proposed to be as high as 30% (Lowenfels et al. 1997). The temporal proximity between diagnoses of chronic pancreatitis and pancreatic cancer raises the risk. This can be explained by misdiagnoses among pancreatitis patients who within 1-2 years are diagnosed with pancreatic cancer (Lowenfels et al. 1993; Bansal et al. 1995). A connection between cancer and persistent inflammation is seen in other cancers such as gastric cancer and *Helicobacter pylorii*, colon cancer and colitis ulcerosa. At the

molecular level a key role in the process that links inflammation to carcinogenesis seems to be activation of COX-2.

Type-II diabetic patients have a 2-fold increased risk, whereas type-I diabetes is not associated with pancreatic cancer (Bansal et al. 1995; Everhart et al. 1995; Talamini et al. 1999; Zendehdel et al. 2003; Huxley et al. 2005). Diabetes can be an early manifestation of pancreatic cancer and is present in 60-80% of patients with pancreatic cancer (Schwarts et al. 1978; Permert et al. 1994). In a large meta-analysis from 1966 to 2005 with 17 case-control and 19 cohort studies, individuals with a recent diagnosis (< 4 years) of diabetes had a greater risk of pancreatic cancer compared to individuals with a history of diabetes longer than 5 years (OR 2.1 vs. 1.5; p=0.005) (Huxley et al. 2005). A patient over 50 with new-onset diabetes and no risk factors for type-II diabetes, such as overweight or family history, may have pancreatic cancer (Gullo et al. 1994).

Smoking is the strongest environmental risk factor known to cause pancreatic cancer and it increases the risk from 1.2 to 5.4 fold, compared to non-smokers (Boyle et al. 1996; Hruban et al. 1998; Coughlin et al. 2000; Chappuis et al. 2001; Isaksson et al. 2002). It has been estimated that cigarette smoking causes about 25% of all pancreatic cancers (Silverman et al. 1994). For patients with familial pancreatitis, cigarette smokers have a higher risk for pancreatic cancer which manifests itself 10 years earlier than in non-smokers (Rulyak et al. 2003). Carcinogens, such as those inhaled in smoking, cause damage to pancreatic DNA, which might contribute to genetic mutations and cancer development (Li et al. 2004).

High dietary intake of saturated fat, red meat, salt and carbohydrate have been associated with an increased risk of pancreatic cancer, whereas fruits, vegetables, and dietary fibre have been reported to be protective factors (Howe et al. 1992; Ghadirian et al. 1995). In other reports dietary factors have not been associated with a higher risk (Michaud et al. 2005). Protective effect of fruits may be related to folate and other methyl-donor groups (Stolzenberg-Solomon et al. 2001). Occupational exposure to carcinogens has been suspected as risk factor for pancreatic cancer (Garabrant et al. 1992; Kauppinen et al. 1995; Fryzek et al. 1997).

Pancratic cancer has the highest frequency of K-*ras* mutations (>85%) of all cancers. In pancreatic cancer K-*ras* mutation has been associated with cigarette smoking and alcohol consumption (Malats et al. 1997). Other factors associated with K-*ras* mutation are serum concentrations of organochlorine compounds, organic solvents and DDT (Porta et al. 1999; Alguacil et al. 2002). These studies propose that K-*ras* mutations may be related to lifestyle and environmental factors.

Although causal role of these environmental factors is still to be studied, there is some evidence of DNA damage derived from carcinogen exposure (Kadlubar et al. 1998; Wang et al. 1998; Li et al. 2002). These studies suggest that the pancreatic tissue is vulnerable to carcinogen exposure and DNA damage, which may contribute to genetic mutation, and into cancer development. There is also evidence, that same individuals may have a deficient carcinogen detoxification and DNA repair

capacities and have therefore an increased risk for pancreatic cancer (Duell et al. 2002; Duell et al. 2002).

MOLECULAR PATHOGENESIS

Molecular pathology

Ductal adenocarcinomas are most likely to develop from ductal proliferative lesions arising in the pancreatic duct system. Genetic studies have enabled the identification and classification of morphological precursor lesions of pancreatic ductal adenocarcinoma, i.e. pancreatic intraepithelial neoplasia (PanIN) (Luttges et al. 2000). The pancreatic ductal epithelium progresses from normal ducts to increasing grades of pancreatic intraepithelial neoplasia, to invasive cancer (Hruban et al. 2000). Three grades of PanIN have been distinguished. Pancreatic cancer, like many other malignant diseases, results from the accumulation of acquired mutations of genes, such as oncogenes, tumour-suppressor genes, and genomic maintenance genes. A tumour progression model for pancreatic ductal adenocarcinoma links alterations in ductal epithelia with genes associated with the progression of normal duct epithelium to invasive cancer (Table 2 and 3) (Sohn et al. 2000; Sakorafas et al. 2001). The accumulation of such genes is thought to progress in a predictable time course.

Table 2. Oncogenes and tumour-suppressor genes commonly altered in pancreatic adenocarcinoma.

Gene	Alteration frequency (%)
Oncogenes	
K-*ras*	75-100
HER2/neu	65-70
AKT2[1]	10-20
Tumour-suppressor and genome-maintenance genes	
TP53	40-75
$p16^{INK4a*}$	27-98
$p19^{ARF*}$	27-82
CDKN2B	27-48
SMAD4	50-55
FHIT[2]	66-70
RB1[3]	0-10
BRCA2	7-10

*CDKN2A. Modified from Donghui Li et al. 2004. [1]Arabidopsis K(+) channel; [2]fragile histidine triad; [3]retinoblastoma

The first notable gene mutation identified was K-*ras* oncogene, which is found in 85% of the sporadic pancreatic cancers and it is found at an early stage of pancreatic cancer progression (Almoguera et al. 1988). Another oncogene, HER-2/neu (erbB2) and its amplification, is also one of the earliest alterations since it is found already in PanIN-1 lesions.

The second step of pancreatic cancer progression includes inactivation of tumour-suppressor genes p16, TP53 and DPC4 (MADH4 or SMAD4), that possibly occurs in PanIN-2 and PanIN-3 stages (Wilentz et al. 2000). Alterations of p16, TP53 and DPC4 are found in 82-95, 76 and 53% of pancreatic cancers (Caldas et al. 1994; Rozenblum et al. 1997; Schutte et al. 1997). Inactivation of tumour suppressor gene BRCA2 is restricted

to PanIN-3 and invasive carcinoma lesions and probably represents a late step in the course of tumourigenesis. BRCA2 alteration is found in 10% of pancreatic cancers (Rozenblum et al. 1997).

There are several systematic analyses to identify gene expression in pancreatic cancer, which reveal dozens of new gene alterations (Zhou et al. 1998; Crnogorac-Jurcevic et al. 2002). Their clinical value has to be evaluated further, but may in the future generate new serum markers for clinical use (Zhou et al. 1998).

Table 3. A progression model of pancreatic cancer: gene mutations

	Alteration frequency (%)				
Histology	**K-*ras***	**TP53**	**HER-2/neu**	**p16^{INK4a}**	**SMAD4**
Normal pancreas	rare	0		0	
PanIN1A	20	0		30-33	
PanIN1B		0-35		27	
PanIN2		9-20		55	
PanIN3	75	12-75	found	71	
Ductal adenocarcinoma	80-90	4-76	10	40-100	53-55

Moskaluk et al. 1997; Luttges et al. 1999; Hruban et al. 2001; Schneider et al. 2003; Li et al. 2004

Molecular biology

The genetic alterations are well studied in pancreatic cancer, but the implications on biological behaviour of pancreatic cancer are poorly understood. The aggressive nature of pancreatic cancer is thought to be related to mutation and inactivation of oncogenes and tumour-suppressor genes, as well as abnormal expression of a variety of proteins and receptors involved in tumour growth, differentiation and migration (Li et al. 2004). Pancreatic cancer overexpresses growth factors and their receptors, such as epidermal growth factor family, vascular endothelial growth factor, fibroblast growth factor, and cytokines, such as transforming growth factor β, interleukin 1, interleukin 6, interleukin 8, and tumour necrosis factor α (Yamanaka et al. 1993; Korc 1998; Saito et al. 1998; Kleeff et al. 1999; Luo et al. 2001; Shi et al. 2001). Overexpression of growth factors and disturbance of growth inhibitory factors contribute to inhibition of programmed cell death, self-sufficiency in growth signals, angiogenesis, and metastasis (Li et al. 2004). Expression of endothelial growth factor is regulated mainly by hypoxia. Interleukin 8 and endothelial growth factor are main angiogenic factors in pancreatic cancer (Shi et al. 2001). Pancreatic tumours have a dense vessel network in the periphery and avascular areas in the center (Mäkinen et al. 2000). High production of vascular endothelial growth factor and interleukin 8 is also seen at the periphery of tumours, where there is no hypoxia. This suggests that there are other factors that effect on production. Exogenous factors such as hormones, cytokines, and growth factors modulate expression of endothelial growth factor thereby affecting angiogenesis (Neufeld et al. 1999).

Genetic alterations, such as activation of oncogenes and inactivation of tumour suppressor genes and upregulation of inducible proteins, such as cytokines and growth factors contribute to downstream gene's expression and thereby to tumour cell cycle and cell survival alteration, adhesion, invasiveness and angiogenesis (Li et al. 2004).

DIAGNOSIS AND MANAGEMENT

Diagnosis and preoperative staging

Symptoms and signs

Early symptoms of pancreatic cancer, such as epigastric discomfort are often non-specific. Symptoms of a tumour in the head of the pancreas are generally caused by compression of the bile duct, the pancreatic duct, the duodenum, mesenteric and celiac nerves. Tumours in the left portion of the pancreas become symptomatic later in the course of the disease (Büchler et al. 2004). Painless jaundice may be the only sign of a small pancreatic-head tumour, whereas back pain may be a sign of tumour growth into the retroperitoneal space and splanchnic plexus. Pancreatic cancer may manifest as type-II diabetes mellitus, malabsorption or acute pancreatitis, the latter caused by obstruction of the pancreatic duct (Kalser et al. 1985; Lin et al. 1990). These symptoms bring patients to physician and pancreatic mass may be diagnosed by imaging procedures, such as computer tomography (Li et al. 2004).

Imaging

For patients with symptoms of pancreatic cancer a diagnostic work-up is generally accepted and include CT as the first line procedure (DiMagno et al. 1999; Alexakis et al. 2004; Li et al. 2004). Abdominal ultrasonography is often the initial screening technique for abdominal pain due to its low cost and easy availability. However, it has low sensitivity and specificity of 67% and 40%, in the diagnosis of pancreatic cancer (Rosch et al. 1992). Although, a Japanese group has shown remarkably high sensitivity and specificity even for small tumours (Tanaka et al. 1996). Contrast enhanced CT, using multislice scanners with arterial and portal venous phases of contrast enhancement, is currently the most useful imaging technique (Freeny et al. 1993). Tumour growth to adjacent organs, such as portal vein, tumour size and location as well as possible distant metastases can be detected by this modality in 80 to 90% of patients (Chong et al. 1998; McCarthy et al. 1998). Limitations of CT include false positive results caused by inflammatory masses, missed small pancreatic tumours, missed nodal and small hepatic metastastases as well as missed peritoneal and omental spread (Scaglione et al. 2005). Sensitivity of dual phase spiral CT is related to tumour size; the sensitivity for tumours <15mm is 67%, compared with up to 100% for tumours of >15mm (Legmann et al. 1998). Magnetic resonance imaging (MRI) is as accurate as CT for the diagnosis of pancreatic cancer (Semelka et al. 1996).

However, if a patient has an unspecific enlargement of the pancreatic head on CT, then an MRI may demonstrate a definitive mass (Semelka et al. 1996).

Endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreaticography (MRCP) provides additional information on bile and pancreatic ducts (Vitellas et al. 2000). ERCP is recommended to be done after CT scanning, because manipulation of the biliary tree causes inflammatory changes and therefore small tumours may be missed on CT (Li et al. 2004).

Endoscopic ultrasonography (EUS) is highly sensitive in the detection of smaller tumours and invasion of major vascular structures (Rosch et al. 1992). EUS is more specific (91-96%) than CT (66-88%) or US (64-88%) for the diagnosis of tumours in the head of the pancreas (Palazzo et al. 1993; Nakaizumi et al. 1995). Helical CT has been compared with EUS and it seems to be as accurate as EUS except in smaller (<3cm) tumours (Legmann et al. 1998). A major limitation with EUS is its operator dependence.

Novel imaging techniques have been introduced in the last decade to improve preoperative staging and diagnosis, such as positrion emission tomography (PET) and laparoscopic US, but those are not yet in wider clinical use (Freeny et al. 1993; Minnard et al. 1998; Midwinter et al. 1999; Mertz et al. 2000; Freeny 2001).

After imaging procedures have identified a pancreatic mass a tissue sample is recommended if feasible. Due to the anatomic location of the pancreas, biopsies to obtain histological verification are often impossible particularly in cases of small tumours. Fine needle biopsy may be obtained by CT-guided fine-needle aspiration, transabdominal ultrasound-guided fine-needle aspiration, or fine needle aspiration under endoscopic ultrasound guidance (Di Stasi et al. 1998). If the patient seems to have metastatic disease, the biopsy is recommended to be taken from a metastasis, and if positive for adenocarcinoma, it is evidence for metastatic pancreatic cancer (Li et al. 2004).

In summary, imaging of pancreatic cancer involves both diagnosis and staging of the tumour. The variety of imaging modalities available for pancreatic diseases demands a rational for their use. A pancreatic mass may be identified and staged by CT scanning and the diagnosis can be obtained by fine needle tissue sample guided by ERCP, EUS, US or CT. Laparoscopy may be recommended to rule out the presence of small liver or peritoneal metastases for selected patients, who seem to have a resectable disease on the basis of preoperative imaging studies (Jimenez et al. 2000; Li et al. 2004).

Serum markers

Several serum tumour markers have been reported as diagnostic markers, but they have limitations, such as low sensitivity, and specificity (Table 4) (Eskelinen et al. 1999a). Carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA) and carbohydrate antigen 242 (CA 242) are three established markers for pancreatic cancer. When used in combination, they have up to 90% specificity (Haglund et al. 1986; Haglund et al. 1989; Haglund et al. 1994; Ni et al. 2005).

CA 19-9 is the most widely used marker for pancreatic cancer. Values >37 U/ml are considered positive and are measured in 78-87% of pancreatic cancer patients (Haglund et al. 1986; Safi et al. 1986). Five to 10% of the population have the Lewis negative blood group (Haglund et al. 1986; Lamerz 1999) and do not therefore have detectable serum CA 19-9 level even with metastatic or recurrent pancreatic cancer (Narimatsu et al. 1998). However, approximately 20% of patients are CA 19-9 negative at the time of pancreatic cancer diagnosis (Haglund et al. 1986; Berger et al. 2004). In some pancreatic cancer patients, Lewis negativity may be mistyped due to circulating antibodies (Hirano et al. 1987). However, after resection of the pancreatic tumour these false Lewis negative patients return to their true Lewis status and can be followed with CA 19-9 values post-operatively (Berger et al. 2004).

In jaundiced patients with benign hepatobiliary or pancreatic disease CA 19-9 may be false-positive (>37 U/ml) in 20-43% of the patients (Sawabu et al. 1986; Sawabu et al. 2004; Ni et al. 2005). However, CA 19-9 values >500 U/ml are less common in benign diseases (Haglund et al. 1986). In non-jaundiced patients values >100 U/ml are highly suggestive for pancreatic cancer (Haglund et al. 1986).

CEA is the most commonly used tumour marker for gastrointestinal tumours, especially for monitoring colorectal cancer patients (Roder 2001). In pancreatic cancer, elevated serum CEA (>5µg) has sensitivity of 46-60% and specificity of 57-75% (Pasanen et al. 1992; Louhimo et al. 2004; Ni et al. 2005). In benign hepatobiliary or pancreatic diseases CEA may be positive in 24% of the patients (Sawabu et al. 2004).

CA 242 (>20 kU/l) has lower sensitivity (68-74%), but higher specificity (76-91%) when compared to CA 19-9 (Pasanen et al. 1992; Haglund et al. 1994; Louhimo et al. 2004; Ni et al. 2005). Tumour markers may be used in parallel to obtain better diagnostic accuracy. For example, combination of CEA and CA 242 increased the specificity to 92% in the diagnosis of pancreatic cancer in one study (Ni et al. 2005).

Table 4. Insidence of various serum tumour markers in patients with pancreatic cancer or benign hepatobiliary and pancreatic diseases.

	Pancreatic cancer (%)	Benign diseases (%)
CA 19-9	78-87	22
CEA	46-60	24
CA 242	68-74	7-15
CA 72-4	38-39	1
CA 125	45-66	13-35
CA-50	71-76	28
DUPAN-2	66	36
SLX *	53	6
ST-439 **	56	7
s-hCG	63	

Haglund et al. 1986, 1994; Louhimo et al. 2004; Sawabu et al.2004.

*sialyl difucosyl Le^x antigen, **sialyl Le^x-Tn antigen, ***free beta subunit of human chorionic gonadotropin

Management

Role of pancreatic and biliary endoscopy

After the anatomy of the tumour in relation to surrounding organs is defined by CT, endoscopic retrograde cholangiopancreatography (ERCP) and stent placement in cases of obstructive jaundice can be performed (Brugge et al. 1999; Pisters et al. 2001). In cases where tumours appear to be locally advanced or metastatic, an expanding metal stent is recommended for durability, compared with plastic stents (Davids et al. 1992). This is, however, not without controversy: there are studies that implicate preoperative biliary stenting increases postoperative morbidity (Sherr 1994; Povoski et al. 1999; Sewnath et al. 2002). In two large studies at the Johns Hopkins and Memorial Sloan-Kettering, there were slightly more fistulae and infections in the stent group, but there were no effects on overall morbidity and mortality (Lygidakis et al. 1987; Sohn et al. 2000). Symptomatic jaundiced patients may not be able to go without biliary drainage before definitive surgery due to symptoms or liver dysfunction, and therefore ERCP with stent placement for jaundiced and symptomatic patients in an experienced centre is advised (Lillemoe 1999). In addition, ERCP provides information on bile and pancreatic ducts and it can be used to obtain brush cytology. Preoperative cytology diagnosis by brushing during ERCP is highly specific (98%), although sensitivity is relatively poor (60%) (Stewart et al. 2001).

Surgical treatment

Surgery is the only curative treatment for pancreatic adenocarcinoma. Radical surgery with curative intent is possible in 15-20% of the patients, and the 5-year survival rate after radical resection is 7-26% (Tsao et al. 1994; Nitecki et al. 1995; Yeo et al. 1995; Capussotti et al. 2003; Cleary et al. 2004; Kuhlmann et al. 2004; Wagner et al. 2004). Resectable pancreatic cancer is defined, based on preoperative imaging, as a pancreatic tumour without evident involvement of the superior mesenteric artery, coeliac axis, or the superior mesenteric-portal venous confluence, and no evidence of metastasis (Li et al. 2004). Factors that do not rule out a curative treatment, include direct tumour invasion into duodenum, stomach, or colon, lymph node metastasis within the operation fields, venous impingement or minimal invasion of the superior mesenteric vein – splenic vein – hepatic portal vein trifurcation or gastroduodenal artery encasement (Alexakis et al. 2004).

Codivilla in 1898 and Kausch in 1909 were the first to successfully perform partial pancreatoduodenectomy (Howard 1999; Specht et al. 2001). Whipple et al. described in 1935 a two-stage procedure for the excision of carcinoma of ampulla of Vater (Whipple et al. 1935). Since then, modifications of the Whipple operation have been introduced, of which Kausch-Whipple modification has become the operation of choice for the management of patients with resectable tumour of the head of the pancreas (Yeo et al. 1999). Kauch-Whipple procedure has been described as a pancreaticoduodenectomy with or without pylorus, and with nodal dissection en block from the anterior and posterior surface of the head of the pancreas, along the superior mesenteric artery and from the

hepaticoduodenal ligament (Jones et al. 1999). Results of pylorus-preserving and standard pancreaticoduodenectomy have been somewhat similar in large series (Williamson et al. 1993; Zerbi et al. 1995; Seiler et al. 2000). Pylorus-preserving procedure has been associated with late gastric emptying (Lin et al. 1999). In one study patients in the pylorus-preserving group returned to work earlier than in the standard Whipple group (Seiler et al. 2000). Conclusions from these studies indicate that pylorus-preserving and classic pancreaticoduodenectomy approaches a similar morbidity and mortality (Roder et al. 1992; Williamson et al. 1993; Yeo et al. 1995; Zagzag et al. 1996; Yeo et al. 1997; Seiler et al. 2000; Stojadinovic et al. 2003; Jacobs et al. 2004; Tran et al. 2004).

The role of extended lymphadenectomy has been studied in three prospective randomised studies, in a prospective non-randomised German study and in a retrospective Japanese study (Mukaiya et al. 1998; Pedrazzoli et al. 1998; Henne-Bruns et al. 2000; Yeo et al. 2002; Farnell et al. 2005). In a randomised multicentre trial of 81 patients in Italy the median survival for the conventional group was 11.2 months with 3- year survival of 8% and in the radical lymphadenectomy group a median survival of 16.7 months with 3- year survival of 8% (no significant difference). In a *post hoc* subgroup analysis node-positive patients had significantly better survival in the radical lymphadenectomy group (Pedrazzoli et al. 1998). In another randomised trial with 146 patients from Johns Hopkins group there was no survival benefit of extended lymphadenectomy, but overall complication rate was significantly higher in that group, when compared to the standard procedure (Yeo et al. 2002). In the third prospectively randomised study, from the Mayo Clinic, with 134 patients, there was no survival benefit for patients that received an extended lymphadenectomy, when compared to conventional pancreaticoduodenectomy (Farnell et al. 2005). According to these studies there is no evidence of survival benefit in extended lymphadenectomy for pancreatic cancer patients.

An extended resection, i.e. superior mesenteric vein or hepatic portal vein resection, in order to achieve a tumour free (R0) result seems to be beneficial and does not appear to increase operative mortality or morbidity (Leach et al. 1998; Bachellier et al. 2001). Resection in the presence of extensive hepatic portal vein encasement in which R0 resection is not achieved is rarely justified, because extended resections are associated with increased morbidity and mortality (Sasson et al. 2002).

In the case of resectable tumours of the body and tail of the pancreas, a left pancreas resection (distal pancreatectomy) is the treatment of choice (Büchler et al. 2004). Total pancreatectomy is rarely justified as it has no survival benefit over standard Kausch-Whipple resection and it has a high risk of morbidity (Launois et al. 1993).

Surgical palliation

Most patients with pancreatic cancer have advanced disease and do not benefit from panreaticoduodenectomy. Advanced pancreatic cancer may cause gastric outlet obstruction or biliary obstruction. To address these symptoms, patients may be treated by surgical or endoscopic palliation. Gastric outlet obstruction has traditionally been managed with open gastrojejunostomy and biliary

obstruction by hepaticocejunostomy. There after, laparoscopic gastrojejunostomy, endoscopic biliary and duodenal stenting have been introduced (Profili et al. 2003; Lopera et al. 2004; Telford et al. 2004; Khan et al. 2005). In general, patients undergoing surgery have more postoperative complications than those who undergo stenting (Raikar et al. 1996; Lopera et al. 2004), although there are patients with locally advanced disease that benefit from surgical bypass (Nieveen van Dijkum et al. 2003). For pain management a chemical coeliac plexus nerve block may be performed (Lillemoe et al. 1993; Rykowski et al. 2000; Abedi et al. 2001).

Adjuvant therapy

The European Study Group for Pancreatic Cancer reported in a prospective randomised trial that adjuvant chemotherapy has a significant survival benefit in patients with resected pancreatic cancer (Neoptolemos et al. 2004). In the USA, adjuvant treatment for resectable pancreatic cancer with fluorouracil-based chemoradiation has been recommended (Gastrointestinal Tumor Study Group 1987; Yeo et al. 1997; Klinkenbijl et al. 1999).

Locally advanced pancreatic cancer is defined as a tumour that encases a vascular structure, (such as the superior mesenteric artery, coeliac axis, or superior mesenteric vein – portal vein confluence), has positive regional lymph nodes, but does not have distant metastasis (Li et al. 2004). Patients with locally advanced and unresectable pancreatic cancer have been recommended to have fluorouracil-based chemoradiation (Moertel et al. 1981). Currently, the standard adjuvant therapies for advanced pancreatic cancer are under debate, but most groups recommend gemcitabine-based treatment (Richards 2005).

Metastatic pancreatic cancer is a progressive state of the disease that is characterized by pain, ascites, anorexia and cachexia (Li et al. 2004). Chemotherapy is never curative for metastatic disease and it provides only very short survival benefit. It may be palliative for pain but its toxic side effects have to be carefully weighed. In a randomised trial, weekly gemcitabine was compared with a weekly fluorouracil for previously untreated patients. Patients that received gemcitabine had a small survival benefit (5.6 vs 4.4 months), but more importantly less disease related symptoms when compared to fluorouracil group (Rothenberg et al. 1996).

Results of surgery

The overall 5-year survival remains extremely low, with quoted values of 0.2-4% for all pancreatic cancer patients (Alanen et al. 1993; Berrino et al. 1998; Faivre et al. 1998; Greenlee et al. 2000; Carpelan-Holmström et al. 2005). According to population based studies only 2.6-14% of patients undergo pancreatic resection (Bramhall et al. 1995; Niederhuber et al. 1995; Hedberg et al. 1998; Sener et al. 1999). Patients who undergo pancreatic resection for non metastatic disease have a 5-year survival of 5-26% and median survival of 11-20 months in different series (Bramhall et al. 1995; Yeo et al. 1995; Carr et al. 1999). Some centralised centres report 5-year survival rates as high as 30-55%

for operable pancreatic ductal adenocarcinoma, but these results are controversial due to the selected patient groups (Conlon et al. 1996; Sohn et al. 2000). Patients who have nonresectable locally advanced disease have a median survival of 6-11 months and those with metastatic disease a median survival of 2-6 months (Alexakis et al. 2004). Surgical mortality rates for the Whipple resection are $\leq$2% in major centres around the world (Trede et al. 1990; Cameron et al. 1993). The best outcomes, in terms of surgical mortality and morbidity, as well as long term survival, are achieved in institutions with most experience (Gordon et al. 1995; Glasgow et al. 1996). Recognised prognostic factors include surgical margin status, grade of differentiation, nodal status, and tumour size (Geer et al. 1993; Yeo et al. 1995)

Future directions

Oncological treatments currently under investigation include combinations of gemcitabine with molecular targeted therapy (Richards 2005). Increasing interest has focused on the molecular biology of pancreatic cancer. Many cellular dysregulates in pancreatic cancer, such as COX-2, epidermal growth factor receptor, mutated K-*ras*, HER2/neu, matrix metalloproteinases (MMP) and various proangiogenic molecules may serve as therapeutic targets (Mason et al. 2001). Cetuximab, an antibody to epidermal growth factor receptor, and trastuzumab, an antibody to HER2, have been combined with gemcitabine in patients with advanced pancreatic cancer (van Cutsem et al. 2004; Xiong et al. 2004). Early results suggest that patients treated with cetuximab have a longer period of stable disease. Another example is an induction of p27, a cell cycle inhibitor, by a pharmacologic inhibition of the MEK signalling pathway (Gysin et al. 2005). MMP inhibitors are drugs that are designed to inhibit degradative enzymes important to cell invasion. However, the first trials have been disappointing (Bramhall et al. 2002). COX-2 is commonly overexpressed in pancreatic cancer and preclinical evidence indicates that selective COX-2 inhibitors have an anti-tumoural activity and that they may improve tumour response to radiotherapy (Kishi et al. 2000; Petersen et al. 2000; Milas 2001). In a phase I clinical study of locally advanced pancreatic cancer, combining celecoxib to gemcitabine and radiotherapy, provided improvement in tumour efficacy but also added toxicity (Crane et al. 2003). However, in a more recent phase II trial celecoxib added to gemcitabine did not increase toxicity (Ferrari et al. 2005).

PROGNOSTIC FACTORS

Favorable prognostic factors are negative resection margins, negative lymph node status, well or moderately differentiated tumours, a small primary tumour (<2cm), and absence of perineural and macroskopic vascular invasion (Allema et al. 1995; Yeo et al. 1998; Kawesha et al. 2000; Neoptolemos et al. 2001; Lim et al. 2003; Richter et al. 2003; Wagner et al. 2004).

TNM classification

The UICC TNM classification is based on the primary tumour extent and size T, nodal status N, and distant metastasis status M. Based on these factors, tumour spread is divided in four stages I – IV. Tumour stage is the gold standard when evaluating the prognosis of a cancer patient. The TNM staging criteria has changed in 1987, 1997 and 2002 as shown in Table 5. For nodal status a minimum of 10 nodes should be investigated. Survival rates between different TNM stages differ significantly ($p<0.001$) (Isaji et al. 2004). In many studies TNM stage has not been stated, but tumour size, nodal status and distant metastasis are available for evaluation (Geer et al. 1993; Tascilar et al. 2001; Kuhlmann et al. 2004; Nitori et al. 2005; Saitou et al. 2005). Small tumour size has been a favorable prognostic factor in several multivariate studies (Geer et al. 1993; Tascilar et al. 2001; Kuhlmann et al. 2004). Tumour size relates to the invasion of lymphatic vessels, large vessels, peripancreatic tissue and adjacent organs and therefore T - tumour status in TNM classification should be more accurate in defining prognosis than the size of the tumour alone.

Nodal status N has been a prognostic factor in multivariate analysis in most studies (Geer et al. 1993; Kuhlmann et al. 2004; Nitori et al. 2005; Saitou et al. 2005), although in some studies this has not been observed (Kayahara et al. 1995; Tascilar et al. 2001). The presence of distant metastasis is defined in the UICC classification as metastasis to distant organs, peritoneum, or distant lymph nodes. In a series of patients resected for cure, M status has little relevance and it has not been an independent prognostic factor in those series (Nitori et al. 2005; Saitou et al. 2005).

Table 5. TNM Clinical Classification of pancreatic exocrine tumours according to UICC.

1987		1997		2002	
		T - Primary Tumour			
TX	Primary tumour can not be assessed	TX	Primary tumour can not be assessed	TX	Primary tumour can not be assessed
T0	No evidence of primary tumour	T0	No evidence of primary tumour	T0	No evidence of primary tumour
		Tis	Carcinoma in situ		
T1a	Limited to pancreas ≤ 2 cm	T1	Limited to pancreas ≤ 2 cm	T1	Limited to pancreas ≤ 2 cm
T1b	Limited to pancreas > 2 cm				
T2	Tumour extends to: duodenum, bile duct, peripancreatic tissue	T2	Limited to pancreas > 2 cm	T2	Limited to pancreas > 2 cm
T3	Tumour extends to: stomach, spleen, colon, adjacent large vessels	T3	Tumour extends to: duodenum, bile duct, peripancreatic tissue	T3	Beyond pancreas
		T4	Tumour extends to: stomach, spleen, colon, adjacent large vessels	T4	Coeliac axis or superior mesenteric artery
		N - Regional lymph nodes			
NX	Regional lymph nodes not assessed	NX	Regional lymph nodes not assessed	NX	Regional lymph nodes not assessed
N0	No regional lymph node metastasis	N0	No regional lymph node metastasis	N0	No regional lymph node metastasis
N1	Regional lymph node metastasis	N1	Regional lymph node metastasis	N1	Regional lymph node metastasis
		N1a	Metastasis in a single regional lymph node		
		N1b	Metastasis in multiple regional lymph node		
		M - Distant metastasis			
MX	Distant metastasis not assessed	MX	Distant metastasis not assessed	MX	Distant metastasis not assessed
M0	No distant metastasis	M0	No distant metastasis	M0	No distant metastasis
M1	Distant metastasis	M1	Distant metastasis	M1	Distant metastasis

				Stage Grouping							
				Stage 0	Tis	N0	M0	Stage 0	Tis	N0	M0
Stage 1	T1,T2	N0	M0	Stage 1	T1, T2	N0	M0	Stage 1A	T1	N0	M0
								Stage 1B	T2	N0	M0
Stage 2	T3	N0	M0	Stage 2	T3	N0	M0	Stage 2A	T3	N0	M0
								Stage 2B	T1-T3	N1	M0
Stage 3	AnyT	N1	M0	Stage 3	T1-T3	N1	M0	Stage 3	T4	Any N	M0
Satge 4	AnyT	AnyN	M1	Satge 4a	T4	Any N	M0	Satge 4	Any T	Any N	M1
				Satge 4b	Any T	Any N	M1				

Histological type

Overall 5-year survival of pancreatic ductal adenocarcinoma has been reported to be as low as 0.2% when a careful histologically verified diagnosis has been assessed (Carpelan-Holmström et al. 2005). The phenotypic classification of pancreatic neoplasia is based on cell lineage and can be divided into three categories, ductal, acinar and endocrine. Tumours of ductal origin include ductal adenocarcinoma, intraductal papillary-mucinous neoplasm, mucinous cystic neoplasm, medullar carcinoma and other rare tumours (Table 6) (Hamilton et al. 2000; Luttges et al. 2000). Pancreatic ductal adenocarcinoma is the most common neoplasm of ductal origin and accounts for over 90% of tumours. Variants of pancreatic ductal adenocarcinoma include adenosquamous carcinoma, undifferentiated carcinoma and mixed ductal-endocrine carcinoma, which do not differ biologically from pancreatic ductal adenocarcinoma (Klöppel 2000). Mucinous cystadenocarcinoma and intraductal mucinous carcinomas in situ have a more favourable prognosis after resection and are therefore not considered as variants of ductal adenocarcinoma (Yeo et al. 1997).

Table 6. WHO histological classification of epithelial tumours of the exocrine pancreas.

Benign
Serous cystadenoma
Mucinous cystadenoma
Intraductal papillary-mucinous adenoma
Mature teratoma
Borderline (uncertain malignant potential)
Mucinous cystic neoplasm with moderate dysplasia
Intraductal papillary-mucinous neoplasm with moderate dysplasia
Solid-pseudopapillary neoplasm
Malignant
Ductal adenocarcinoma
Mucinous noncystic carcinoma
Signet ring cell carcinoma
Adenosquamous carcinoma
Undifferentiated (anaplastic) carcinoma
Undifferentiated carcinoma with osteoclast-like giant cells
Mixed ductal-endocrine carcinoma
Serous cystadenocarcinoma
Mucinous cystadenocarcinoma
non-invasive
invasive
Intraductal papillary-mucinous carcinoma
non-invasive
invasive (papillary-mucinous carcinoma)
Acinar cell carcinoma
Acinar cell cystadenocarcinoma
Mixed acinar-endocrine carcinoma
Pancreatoblastoma
Solid-pseudopapillary carcinoma

Histological differentiation

For ductal adenocarcinomas, grade or histological differentiation according to the criteria of the World Health Organisation (WHO) are independent prognostic factors in several studies (Table 7) (Geer et al. 1993; Tascilar et al. 2001; Kuhlmann et al. 2004; Nitori et al. 2005).

Table 7. Histological differentiation according to criteria of the WHO.

Tumour grade	Glandular differentiation	Mucin production	Mitosis / 10 HPF	Nuclear atypia
1	Well-differentiated	Intensive	5	Little polymorphism polar arrangement
2	Moderately differentiated duct-like structures and tubular glands	Irregular	6 - 10	Moderate polymorphism
3	Poorly differentiated glands, mucoepidermoid and pleomorphic structures	Abortive	10	Marked polymorphism and increased nuclear size

Age, time period and gender

In most studies age and gender are not significant prognostic factors for patients with pancreatic cancer (Geer et al. 1993; Kuhlmann et al. 2004). In a multicenter study of 31,323 pancreatic cancer patients there were no differences in survival between patients operated during different time periods (1978-80 vs. 1987-89) (Faivre et al. 1998). In single institution studies, a significant improvement has been shown over time (Yeo et al. 1995), this is probably due to centralisation of pancreatic surgery and improved intra- and postoperative care (Gouma et al. 1999; Neoptolemos 2002).

Treatment associated factors

The most important treatment-associated factor is the complete resection of the tumour whereby no micro- or macroscopic residual tumour remains (Carr et al. 1999; Hermanek 1999). The possibility for resection is highly associated with tumour stage and therefore in multivariate analysis, either surgery for cure or tumour stage is an independent prognostic factor. For long term survival performing surgery for cure is essential: patients resected for cure and patients given supportive care had one year survival rates of 30 and 2%, respectively (Bramhall et al. 1995). Results of extended resection in order to obtain complete resection even in advanced disease have been favourable for survival. However, there does not seem to be any indication for extended resection if a tumour free result (R0) is not achievable (Sasson et al. 2002; Capussotti et al. 2003; Shoup et al. 2003; Hartel et al. 2004; Nakao et al. 2004).

Survival duration may be prolonged in patients who receive preoperative chemoradiation (Lim et al. 2003; Joensuu et al. 2004). The overall median survival for patients with locally advanced disease

treated with chemoradiation was 11 months, while it was 6.5 months without multimodal therapy (p=0.004) (Kim et al. 2002). There are few case reports of pancreatic cancer patients deamed unresectable at the time of exploratory laparotomy, but subsequently underwent successful resection after gemcitabine-radiotherapy (Ammori et al. 2003). However, disease recurrence following neoadjuvant therapy and potentially curative pancreaticoduodenectomy remains common (Kim et al. 2002).

Serum markers

Serum levels of CA 19-9 predict recurrence and survival (Glenn et al. 1988; Sperti et al. 1993; Montgomery et al. 1997; Ikeda et al. 2001). A CA 19-9 serum level that decreases to normal levels (<37kU/l) after resection was associated with longer survival compared to those whose CA 19-9 level did not normalize (Glenn et al. 1988). A secondary elevation of CA 19-9 during post-operative follow-up preceded detection of recurrence with a CT scan by 2 to 9 months (Tian et al. 1992). CA 19-9 is a useful predictor of response to gemcitabine in patients with advanced pancreatic cancer (Halm et al. 2000). Five to 7% of patients with Lewis negative blood group have undetectable CA 19-9 serum levels. In other patients a low serum level of CA19-9 correlates with better survival (Berger et al. 2004).

CA 242, which is closely related to CA19-9, has been shown to be an independent prognostic marker (Lundin et al. 1995; Louhimo et al. 2004; Ni et al. 2005), while CA 72-4 and hCGβ were reported to be stronger prognostic factors than CEA, CA 19-9 and CA 242 (Louhimo et al. 2004). However, an earlier study showed that CA 242 yielded more prognostic information than CA 19-9 (Lundin et al. 1995).

Potential new markers

Several novel and promising prognostic tissue markers have been reported (Eskelinen et al. 1999b). New and promising markers, their integration to tumour development and their prognostic significance are presented in the following text.

Oncogenes and tumour suppressor genes

Inactivation of tumour suppressor genes DPC4, p16 and p53 (Hahn et al. 1996) and activation of dominant oncogenes K-ras and Cyclin D1 play a critical role in the tumourigenesis of pancreatic cancer and thereby have been proposed as prognostic factors for pancreatic cancer.

K-*ras* belongs to the family of proto-oncogenes that encode GTP-ase activity. Growth and differentiation signals are transduced by K-*ras* to activate protein kinases such as cyclin kinases. Between 75-90% of tumours exhibit a point mutation of the K-*ras* oncogene and approximately 50% of these tumours express the K-*ras* protein (Shibata et al. 1990; Lemoine et al. 1992). Certain K-*ras* mutation subtypes have been associated with poor prognosis (Conlon et al. 1996).

Around 55% of pancreatic cancer patients bear a mutation of the tumour suppressor gene SMAD4, also termed DPC4. In a study of 249 patients operated on for pancreatic adenocarcinoma, those with tumour expression of SMAD4 protein had longer survival (median 19 months) than patients whose tumours did not express SMAD4 protein (15 months) (Tascilar et al. 2001).

p53 controls the entry of cells into the cell cycle and has a role in initiating DNA repair (Kastan et al. 1991). Approximately 65-85% of pancreatic cancers have a point mutation of p53 (Barton et al. 1991). p16 plays a key role in controlling the G1 checkpoint of the cell cycle. Loss of p16 expression occurs in up to 95% of pancreatic cancers. However, no association of p16 and p53 with prognosis has been observed in larger studies (Zhang et al. 1994; Lundin et al. 1996; Kawesha et al. 2000; Dong et al. 2003), although a series of smaller studies have found prognostic significance (DiGiuseppe et al. 1994; Gerdes et al. 2002).

Cyclin D1 is a cell cycle regulator that may act as an oncoprotein and is overexpressed in 65% of tumours. It has been associated with shorter survival in a small study of 82 patients, although it was not independent of tumour stage or grade (Gansauge et al. 1998). In a larger study of 142 patients Cyclin D1 was not considered to be a prognostic factor (Kawesha et al. 2000).

Apoptosis is a normal process of programmed cell death and regulates homeostasis in normal tissues. The bcl-2 family of apoptotic genes includes, among others bcl-2, bcl-x, bax and bac. Bcl-2, bcl-x and bax have been favourable indicators of prognosis in small patient series (Mäkinen et al. 1998; Evans et al. 2001; Dong et al. 2005), but also opposite results have been reported (Friess et al. 1998; Nio et al. 2001).

p27

The p27/Kip1 protein belongs to the family of proteins called cyclin-dependent kinase inhibitors (CDKIs). These proteins play an important role as negative regulators of cell cycle-dependent kinases during the progression of the cell cycle (Sherr et al. 1995). p27 regulates the progression from G1 into S phase by binding to and inhibiting the cyclin E/Cdk2 complex, which is required for entry into S phase. It also interacts with various other cyclin complexes and is therefore designated as a universal CDKI (Lloyd et al. 1999). The expression is regulated by cell contact inhibition and by specific growth factors, such as transforming growth factor beta (TGF-β). Since CDKs can inhibit cell proliferation, they have a role as tumour suppressor genes. Reduced expression of the p27/Kip1 protein has been reported in several human tumours and it has been associated with high tumour grade and increased mortality in cancer of the breast, lung, prostate and bladder (Esposito et al. 1997; Porter et al. 1997; Yang et al. 1998; Del Pizzo et al. 1999). p27 has also been a promising novel prognostic marker in colorectal (Loda et al. 1997) and gastric cancer (Kwon et al. 1999). However, there is limited information on p27 and the clinical outcome of pancreatic cancer patients.

Molecular markers

Growth factors

Pancreatic cancer demonstrates expression of numerous growth factors and their receptors, which possibly contribute to tumour cell growth. Several studies have shown that high level of epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), and transforming growth factor alfa (TGFα) are correlated with reduced patient survival (Yamanaka et al. 1993; Uegaki et al. 1997). The c-erbB´s-2, 3, and 4 belong to the same family of type 1 growth factor receptors as EGFR, but their prognostic significance in pancreatic cancer is yet to be determined (Carraway et al. 1994; Friess et al. 1995; Graber et al. 1999; Kawesha et al. 2000). TGFβs are a family of cytokines that have influence on cell division, cell death and cellular differentiation. The three isoforms of TGFβs are shown to be over-expressed and to correlate with survival in a study of 60 pancreatic cancer patients (Friess et al. 1993). Fibroblast growth factors (FGFs) are known to influence cell differentiation, tissue homeostasis, tissue regeneration and angiogenesis and are over-expressed in pancreatic cancer compared to normal tissue at mRNA and protein levels (Yamanaka et al. 1993; Vickers et al. 1999). However, the studies on growth factors and their receptors have only been done on a small number of patients and the results are inconsistent. The prognostic significance of these factors requires further validation in larger groups of patients.

TF

Tissue factor (TF) is a transmembrane glycoprotein that is involved in blood coagulation and intracellular signalling pathways. Patients with malignant diseases are predisposed to hypercoagulation and have higher risk of thrombosis (Valente et al. 2000). This hypercoagulation state is associated with elevated TF expression in several malignancies. In patients with resectable pancreatic cancer, high TF expression, was associated with poor prognosis ($p=0.0076$, hazard ratio 2.0) (Nitori et al. 2005).

Mucins

Mucins are high molecular weight glycoproteins that are produced by various epithelial cells. They are categorised as membrane associated mucins (MUC1, MUC3, MUC4, MUC12 and MUC17), gel forming mucins (MUC2, MUC5 and MUC6), and soluble mucins (MUC7) (Moniaux et al. 2001). MUC4 acts as an intra-membrane ligand to ErbB2. Saitau et al. showed that high MUC4, but not MUC1, expression was associated with poor outcome in patients operated on for pancreatic adenocarcinoma ($p=0.012$, hazard ratio 2.0) (Saitou et al. 2005).

Syndecan-1

Syndecan-1 belongs to the syndecan family of cell surface transmembrane heparane-sulphate proteoglycans (HSPGs). Syndecan-1 has been shown to bind various extracellular matrix (ECM) proteins, such as interstitial collagen, tenascin, fibronectin and basic fibroblast growth factor (Koda et al. 1985; Saunders et al. 1988; Salmivirta et al. 1991; Salmivirta et al. 1992). Through binding to ECM

molecules and growth factors, syndecan-1 is thought to participate in a variety of biological processes, such as cell adhesion, cell matrix interactions, cell proliferation, cell migration, coagulation cascades and infection of cells with microorganisms (Woods 2001). Four syndecans, syndecan-1, -2, -3 and -4, have been characterised, each with a distinct structure and pattern of expression (Bernfield et al. 1992). All adhesive cells express at least one syndecan, and most express multiple syndecans (Kim et al. 1994). Syndecan-1 is mainly expressed on epithelial cells, but it is also found on mesenchymal cells during development, in pre-B lymphocytes and mature plasma cells (Sanderson et al. 1989; Vainio et al. 1989). Cell-cell interactions are vital for the maintenance of normal tissue architecture and for normal embryonic development. It is thought that high levels of syndecan-1 correlate with the maintenance of epithelial morphology, anchorage-dependent growth and inhibition of invasiveness (Kato et al. 1995; Dhodapkar et al. 1998; Bayer-Garner et al. 2001).

In general, the expression of syndecan-1 mRNA is down-regulated in gastrointestinal malignancies, but in pancreatic cancer it seems to be upregulated (Conejo et al. 2000). The loss of expression of epithelial syndecan-1 has been associated with both shorter overall survival and a recurrence-free survival in squamous cell carcinoma of the head and neck, lung and gastric cancer (Inki et al. 1994; Anttonen et al. 2001; Wiksten et al. 2001). In a recent study, the expression of syndecan-1, was evaluated separately in epithelia and stroma of breast carcinoma and found to be induced in the stroma of infiltrating breast carcinoma (Stanley et al. 1999). In a gastric cancer study of syndecan-1 expression it was associated with longer survival, while stromal syndecan-1 expression was associated with shorter survival (Wiksten et al. 2001).

Tenascin C

Tenascin is a large (180 – 300 kDa) hexameric glycoprotein located mainly in the extra- cellular matrix (ECM). It is a member of an adhesion-modulating family of the ECM. It is expressed transiently during fetal development and present only in restricted locations in normal adult tissue (Erickson et al. 1989). It is sparsely expressed in the areas of basement membrane of skin, colon mucosa and ductal salivary glands (Schalkwijk et al. 1991; Soini et al. 1992; Hanamura et al. 1997). It is seen in the vessel walls of different organs and in visceral smooth muscle (Koukoulis et al. 1991). Tenascin is also expressed in healing wounds, atherosclerosis and hyperproliferative skin diseases (Mackie et al. 1988; Schalkwijk et al. 1991; Wallner et al. 1999).

In addition to the surrounding of growing or differentiating epithelia, tenascin-C is prominent in the stroma of a variety of tumours (Koukoulis et al. 1991). Interactions between tumour cells and ECM are important for tumour invasion and development of metastases (Karademir et al. 2000; Linder et al. 2001). The expression of tenascin-C in tumour stroma suggests altered cell-matrix interactions, which may facilitate epithelial tumour cell invasion and metastases. Tenascin-C levels have been associated with tumour recurrence and prognosis, although the findings are contradictory (Vaidya et al. 1996; Jahkola et al. 1998; Pilch et al. 1999; Juhász et al. 2000; Emoto et al. 2001; Atula et al. 2003). In laryngeal and hypopharyngeal cancers accumulation of tenascin in the blood vessels is an indicator of

unfavourable prognosis. It correlates with metastasis, early tumour recurrence and a lethal outcome (Juhász et al. 2000). In breast cancer the expression of tenascin-C in the invasion border of the tumour is a predictor of local and distant recurrence (Jahkola et al. 1998). In colorectal cancer tenascin has been reported to be a predictor of a worse prognosis in two studies (Kressner et al. 1997; Emoto et al. 2001). In contrast, patients with tenascin positive cervical or gastric cancers, had a better prognosis (Pilch et al. 1999; Wiksten et al. 2003).

COX-2

In population based studies the use of non-steroidal anti-inflammatory drugs has been shown to protect from colorectal and possibly some other cancers (Rosenberg et al. 1998; Meier et al. 2002; Sørensen et al. 2003). Cyclooxygenase-2 (COX-2) is an integral membrane protein associated with acute inflammation and is the rate-limiting enzyme in the biosynthesis of prostanoids (i.e. prostaglandins, thromboxanes and prostacyclins). In most tissues COX-2 is not physiologically expressed. However, hormones, cytokines, growth factors and tumour promoters rapidly induce COX-2 expression (Hamasaki et al. 1993; Jones et al. 1993). At the molecular level a key role in the process that links inflammation to carcinogenesis seems to be activation of COX-2, although the intracellular pathways in that process are still largely unknown (Konturek et al. 2005).

Increased tissue levels of COX-2 are found in several human carcinomas. It is thought that in tumourigenesis COX-2 is involved in proliferation, inhibition of apoptosis and invasion by enhancing production of matrix metalloproteinases (MMP´s) and by promoting angiogenesis (DuBois et al. 1996; Tsujii et al. 1997; Tsujii et al. 1998). Elevated COX-2 expression is associated with poor prognosis in oesophageal, gastric, colon, breast and ovarian carcinomas (Sheehan et al. 1999; Buskens et al. 2002; Ristimäki et al. 2002; Shi et al. 2003; Erkinheimo et al. 2004). In pancreas COX-2 is expressed in the cytoplasm of ductal tumour cells but not in the surrounding stroma (Tucker et al. 1999). In pancreatic ductal adenocarcinomas COX-2 is upregulated in 67-90% (van Rees et al. 2003).

MMP-2

Pancreatic ductal adenocarcinoma is known for exceptionally rapid local tumour invasion and early systemic dissemination that precludes radical surgery (DiMagno et al. 1999). It is known to have a strong desmoplastic stromal reaction around the tumour cells (Binkley et al. 2004). Degradation of the ECM by different proteases is an essential step in tumour cell penetration into adjacent tissues and vessels thereby facilitating metastasis to distant organs (Keleg et al. 2003). MMPs play an important role in tumour invasion by ECM degradation (Kleiner et al. 1999). In cancer research much interest has been devoted to a gelatinase subgroup of MMPs that include MMP-2 (gelatinase A, a 72kD collagenase type IV). MMP-2 is expressed in the membrane of invasive tumour cells and is thought to contribute to pericellular space degradation and to render cancer cells more invasive at the migration front. MMP-2 is known to degrade type IV collagen which is the major component of epithelial basement membranes (Birkedal-Hansen 1995).

In addition to their ability to destroy basement membranes and ECM, MMPs are involved in other functions of tumour progression. It is thought that MMP-2 is needed for proper angiogenesis. Tumour cells introduced into MMP-2 knockout mice have a reduced growth capacity and are less vascularized than tumours in control mice (Itoh et al. 1998). The application of synthetic inhibitors of MMP activity reduces neovasularisation (Stetler-Stevenson 1999). In addition to matrix components, MMPs can cleave other substrates during tumour progression. For example, specific cleavage of laminin-5 by MMP-2 was shown to induce migration of breast epithelial cells (Giannelli et al. 1999). Furthermore, receptors for growth factors might be targeted for proteolysis. The fibroblast growth factor type I receptor (FGFR1), has been identified as a substrate of MMP-2. FGFR1 may be a specific target for MMP2 on the cell surface, yielding a soluble FGF receptor that may modulate the mitogenic and angiogenic activities of FGF (Levi et al. 1996).

MMPs are upregulated and related to tumour relapses and poor survival in a variety of malignancies (Allgayer et al. 1998; Talvensaari-Mattila et al. 2003; Leppä et al. 2004; Torng et al. 2004). Normal pancreas does not express MMP-2, but it is up-regulated in pancreatitis and in pancreatic cancer (Bramhall et al. 1997). In pancreatic cancer MMP-2 is secreted from tumour cells and from stromal cells, although the expression is more dominant in the stromal cells (Gress et al. 1995; Määttä et al. 2000). In pancreatic cancer MMP-2 expression is enhanced in cells at the invasive front compared to the centre of the tumour (Kuniyasu et al. 1999). Although, MMP-2 expression in pancreatic cancer has been investigated in several studies by different methods (Gress et al. 1995; Koshiba et al. 1997; Koshiba et al. 1998; Määttä et al. 2000), the prognostic role of MMP-2 is yet to be fully elucidated (Bloomston et al. 2002).

6. AIMS OF THE STUDY

The aims of the study were to evaluate:

- the expression of p27 in pancreatic ductal adenocarcinoma and acute/chronic pancreatitis and the association between immunohistochemical p27 expression and prognosis in patients with pancreatic cancer.

- the expression of syndecan-1 in pancreatic ductal adenocarcinoma, in normal pancreatic tissue and acute/chronic pancreatitis and to investigate the prognostic significance of stromal and epithelial syndecan-1 expression in pancreatic cancer.

- the expression of tenascin C in pancreatic ductal adenocarcinoma and to compare the results with clinicopathological factors.

- the association between COX-2 expression and prognosis and other clinicopathological factors in pancreatic ductal adenocarcinoma.

- the association between MMP-2 and clinicopathological factors in pancreatic ductal adenocarcinoma.

7. MATERIAL AND METHODS

MATERIAL

Patients

Surgical specimens from 216 patients with histologically verified pancreatic adenocarcinoma treated at the Helsinki University Central Hospital from 1974 to 1998 were included in the study. One patient was excluded from the study due to missing clinical data, 48 due to missing histological specimen and 20 due to inadequate sample. Tissue specimens suitable for immunohistochemical evaluation were available in 147 patients for the study on p27 (I), 144 for the study on syndecan-1 (II), 146 for the study on tenascin C (III), 128 for the study on COX-2 (IV) and 127 for the study on MMP-2 (V). Four normal pancreatic tissue samples, 10 acute pancreatitis and 10 chronic pancreatitis samples were also analysed for studies I, II and III.

Survival data

Survival data of the patients were obtained from patient records, the Statistics Finland and the Population Registry. In survival analysis 8 patients were censored: two patients died due to other causes than pancreatic adenocarcinoma (myocardial infarct and femoral neck fracture) and six patients that where alive by the end of the follow up (followed 4.7-21.7 years after operation). Prior to analysis we decided not to censor patients with survival shorter than 30 days after operation as the death may be due to the aggressive nature of the disease as well as to operative morbidity. However, censoring or excluding postoperative deaths did not affect on the results presented on studies I to V: all p-values remained significant. There were five post-operative deaths in the 1970´s, 11 postoperative deaths in the 1980´s and 3 postoperative deaths in 1990´s while the post operative mortality rate was 19, 16 and 6%, respectively, for all patients operated for pancreatic cancer. Post-operative mortality in patients operated for cure during the same period was 16, 13 and 2,8%, respectively.

Stage

Staging was done according to the UICC TNM classification (Sobin et al. 1997). The original staging of the tumours were done between 1974 to 1987 according to the third edition, 1987 to 1997 according to the fourth edition and 1997 to 1998 according to the fifth edition of the TNM classification. We reassessed the tumour stage of the diseases for the study between 2000 and 2001 according to the fifth

edition of the 1997 TNM classification, which was the latest edition at the time. The stage of the disease was based on clinical data, imaging results, operation records and surgical specimens.

Treatment

All patients included in the study underwent surgery, either pancreaticoduodenectomy for cure (n=95), non-radical pancreaticoduodenectomy (n=39), palliative bypass (n=8) or diagnostic laparotomy (n=5). Patients did not receive neoadjuvant treatment. The operation was considered radical if there was no residual tumour, either macro- or microscopic, on the resection margins or elsewhere and was referred to as radical operation (I – III and V) or R0 resection (IV).

METHODS

Histology

Histological specimens were re-evaluated by one pathologist for hematoxylin and eosin and van Gieson staining. The diagnosis of pancreatic carcinoma was confirmed, histological grade reassessed and the most representative sample was chosen for immunohistochemistry.

Staining

For immunohistochemical staining the following antibodies were used: Anti-Kip1/p27 monoclonal (K25020, Transduction Laboratories, Lexington, KY) (I), a commercial mouse monoclonal antibody (Mab) against human syndecan-1 (B-B4; Serotec, Oxford, UK) (II), a tenascin-specific anti-human monoclonal antibody (clone DB7, Biohit Diagnostics Oy, Helsinki, Finland) (III), a COX-2 specific mouse antihuman monoclonal antibody (160112; Cayman Chemical, Ann Arbor, Mi, USA) (IV) and a commercial Mab against human MMP-2 (Ab-4; Neomarkers, Fremont, USA) (V). Four micrometers thick paraffin sections were mounted on slides coated with 3-aminopropyl-triethoxy-silane (APES) (Sigma, St. Louis, MO, USA) and dried for 12-24 hours at 37°C. The sections were deparaffinised in xylene and rehydrated through graded concentrations of ethanol to distilled water.

Sections were then pre-treated in a 700 W microwave oven for 4 x 5 min in 0.3% citrate acid buffer (pH 6.0). Slides were then cooled for 20 min at room temperature and washed in PBS: distilled water solution (1:10). To block endogenous peroxidase the sections were incubated in 0.5% hydrogen peroxide in methanol for 30 minutes. To reduce non-specific staining, the slides were incubated with diluted (1:67) non-immune horse or mouse serum for 15 minutes. The sections were incubated with primary antibody with dilutions of 1:500 (p27), 1:100 (syndecan-1), 1:2000 (tenascin C), 1:200 (COX-2) and 1:200 (MMP-2). Bound antibody was visualised by the avidin-biotin complex immunoperoxidase technique (ABC) (Elite ABC Kit, Vectastain, Vector Laboratories, Burlingame, CA). The peroxidase staining was visualised with 3-amino-9-ethyl-carbazole (Sigma, A-5754) in

acetate buffer containing 0.03% perhydrol. Sections were counterstained in Mayer´s haematoxylin and mounted in aqueous mounting media (Aquamount, BDH, Poole, UK).

In every staining batch a positive control was included that comprised a section of a p27 (I), syndecan-1 (II) or COX-2 (IV) positive colon carcinoma, tenascin C positive breast carcinoma (III) or MMP-2 positive thyroid carcinoma. Sections treated with PBS alone served as negative controls.

Interpretation of immunohistochemical staining

In studies I, II, III and V the stainings patterns were interpreted by one pathologist who was unaware of the clinical outcome of the patients. In study IV two pathologists interpreted COX-2 stainings independently. If the result differed, a consensus score was chosen after a second look.

Study I: The expression of p27 was scored according to the percentage of p27 positive cancer cell nuclei: less than 5% of nuclei positive score 0; 5-10% score 1; 10-50% score 2 and over 50% score 3. The cut-off level for loss of expression was set to 5%. When samples from more than one site of the tumour were available, the lowest percentage of positive nuclei was considered as representative of the tumour.

Study II: The expression of syndecan-1 was evaluated separately in the epithelium and the stroma. The level of epithelial syndecan-1 expression was scored according to the percentage of positively stained cancer cells: negative ($\leq$5% of cells positive), weak (6% to 20% of cells positive), moderate (21 to 60% of cells positive) or strong (>60% of cells positive). The stromal tissue surrounding cancer cells was evaluated and scored likewise: negative ($\leq$5% of area positive), weak (6% to 20% of area positive), moderate (21 to 60% of area positive) or strong (>60% of area positive). The intensity of the stain did not influence the score. As benign controls, 4 normal pancreatic tissue samples were used to assess the cut-off lines for statistical analysis. In normal pancreas the epithelial expression was moderate or strong in the ductal cells and negative in acinar cells. The normal pancreatic stroma did not express syndecan-1. In statistical analysis a 20 % cut off was used for epithelial expression and 5% for stromal expression.

Study III: An extracellular staining reaction in the stroma around the tumour cells was considered positive. The staining was scored as negative ($\leq$5%), weak (over 5 to 20%), intermediate (over 20 to 60%) and strong (>60%) according to the percentage of positively stained area. The cut-off level for statistical analysis of association was set at 20%, which divided the series into two approximately equal sized groups.

Study IV: Two pathologists interpreted the results independently. The level of COX-2 intensity was considered negative if less than 5% of the tumour cells expressed COX-2, weak if 5-10% of the cells were positive, moderate if 10-50% cells were positive and strong if more than 50% of the cells were positive. There were 6 samples that the pathologists had scored with a difference of two categories. Those samples were re-evalued and the consensus score was used for further analysis. For dichotomic analysis a 5% cut off was used for COX-2 positive tumours.

Study V: The expression of MMP-2 was evaluated separately in epithelium and stroma. The level of epithelial MMP-2 expression was scored according to the percentage of positively stained cancer cells: negative (≤5% of cells positive), weak (5% to 10% of cells positive), moderate (10 to 50% of cells positive) or strong (>50% of cells positive). The stromal tissue surrounding cancer cells was evaluated and scored likewise: negative (≤5% of area positive), weak (5% to 10% of area positive), moderate (10 to 50% of area positive) or strong (>50% of area positive). For the statistical analysis cases with a negative or weak expression of MMP-2 were combined into one group MMP-2 negative, whereas patients with moderate or strong staining were combined into the MMP-2 positive group.

Statistical analysis

The chi square test and Fisher's exact test were used, in cases where there were very small-expected frequencies, to test for association between factors. Life tables were calculated according to the Kaplan-Meier product limit method. The disease-specific overall survival was calculated from the date of diagnosis to death from pancreatic cancer, and patients who died of intercurrent causes were censored. The statistical significance of the difference in survival between groups was calculated using the log-rank test or log-rank for trend when appropriate. Multivariate survival analysis was performed with the Cox proportional hazards model. Cox regression was done using a backward stepwise selection of variables and a p-value of 0.05 was adopted as the limit for inclusion of a covariate.

8. RESULTS

SURVIVAL

In univariate analysis surgery for cure ($p<0.0001$), high histological differentiation ($p<0.0001$), non-metastatic disease ($p=0.0001$), less advanced clinical stage ($p=0.002$), tumour location ($p=0.034$) and younger age ($p=0.049$) correlated with longer survival. The association between survival and nodal status approached significance ($p=0.062$). Gender and tumour size were not significantly associated with survival (Figures 1-6 and Table 8).

There was an association between survival and decade of operation ($p=0.03$). The median survival was 7.2 months for patients who were operated between the years 1974 to 1979, 8.6 months for those patients operated between the years 1980 to 1989 and 13 months for patients operated between the years 1990 to 1998. There was no association between the decade of operation and the proportion of radically operated patients. In the subgroup of radically operated patients (n=95) there was a similar tendency of better survival for patients operated in later years, but the difference was not significant.

TABLE 8 Univariate analysis of the relationship between preopertive characteristics and survival of 147 patients with pancreatic cancer.

Clinicopathological variable	Number of patients	%	1-year cumulative survival (%)	95% CI	2-year cumulative survival (%)	95% CI	5-year cumulative survival (%)	95% CI	chi^2	p
Gender										
Female	81	55	46	36 - 57	21	12 - 30	6	1 - 12	0.04	0.83
Male	66	45	44	32 - 56	20	10 - 29	5	0 - 11		
Age (years)										
≤62	74	50	52	41 - 64	23	14 - 33	7	1 - 13	3.9	0.049
>62	73	50	38	27 - 50	18	9 - 27	5	0 - 10		
Tumour location										
Head	130	88	47	39 - 56	23	15 - 30	7	2 - 11	4.5	0.034
Other location	15	10	33	10 - 57	7	1 - 19	0	0 - 0		
Not available	2	1								
TNM stage										
I	27	18	67	49 - 84	33	16 - 51	11	0 - 23	10.1	0.002
II	49	33	47	33 - 61	27	14 - 39	6	0 - 13		
III	29	20	39	21 - 58	18	4 - 32	7	0 - 17		
IV	41	28	32	18 - 46	7	0 - 15	0	0 - 0		
Not available	1	1								
TNM stage										
I-II	76	52	54	43 - 65	29	19 - 39	8	2 - 14	6.8	0.009
III-IV	70	48	35	24 - 46	12	4 - 19	4	0 - 8		
Not available	1	1								
Tumour size										
≤2cm	26	18	68	50 - 87	20	4 - 36	0	0 - 0	3.0	0.082
2-4cm	73	50	51	39 - 62	25	15 - 35	10	3 - 17		
>4cm	33	22	21	7 - 35	12	1 - 23	3	0 - 9		
Not available	15	10								
Lymph node metastasis										
N0	91	62	56	46 - 66	26	17 - 35	7	2 - 12	3.5	0.061
N1	42	29	32	18 - 46	15	4 - 26	7	0 - 15		
Not available	14	10								
Distant metastasis										
M0	136	93	48	40 - 57	22	15 - 29	6	2 - 10	15.0	0.0001
M1	10	7	10	0 - 29	0	0 - 0	0	0 - 0		
Not available	1	1								
Grade of differentation										
1	15	10	80	60 - 100	40	15 - 65	27	4 - 49	15.6	<0.0001
2	92	63	47	37 - 58	24	15 - 32	6	1 - 10		
3	40	27	28	14 - 41	8	0 - 16	0	0 - 0		
Curativity										
Intent to cure	95	65	57	46 - 67	29	20 - 38	9	3 - 14	18.4	<0.0001
Non-curative	52	35	25	13 - 36	6	0 - 12	0	0 - 0		
Operating decade										
1974-1979	27	18	37	19 - 55	15	0 - 1	0	0 - 0	4.0	0.0440
1980-1989	70	48	43	31 - 22	19	10 - 28	6	0 - 11		
1990-1998	50	34	53	39 - 67	27	14 - 39	9	1 - 18		
Radically operated (n=95)										
1974-1979	19	13	47	25 - 70	21	3 - 39	0	0 - 0	2.7	0.0989
1980-1989	40	27	53	37 - 68	28	14 - 41	10	1 - 19		
1990-1998	36	24	66	50 - 82	34	19 - 50	12	1 - 22		

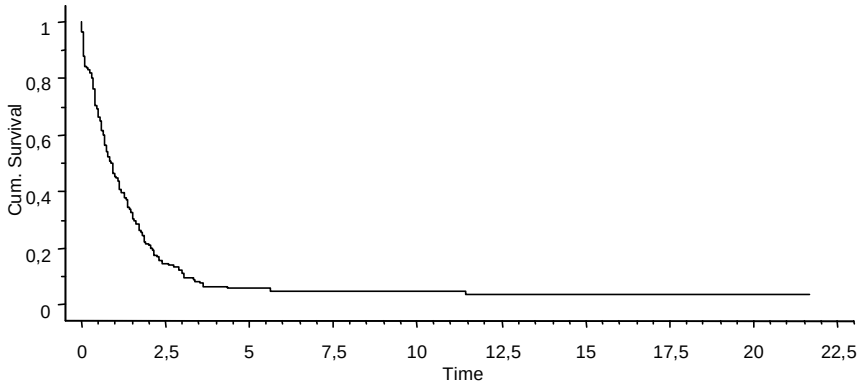


Figure 1 Cumulative survival of 147 patients operated on pancreatic adenocarcinoma in Helsinki University Central Hospital in 1974 - 1998.

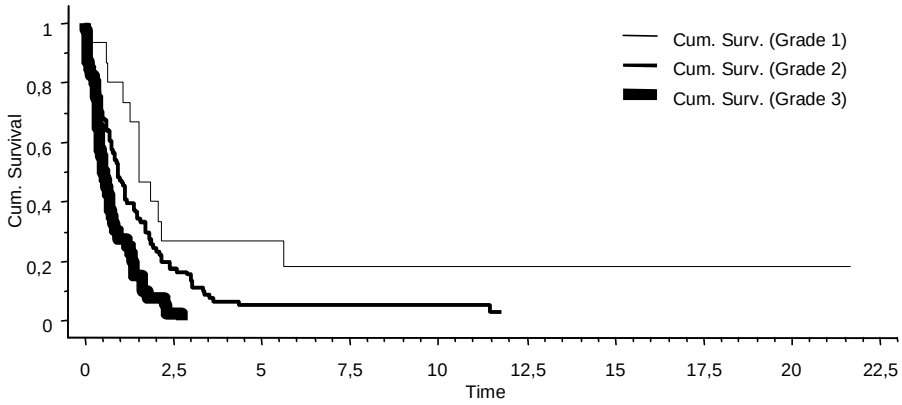


Figure 2 Life tables for Grade 1-3 patients with pancreatic adenocarcinoma. The difference in survival was significant between grades. p<0.0001

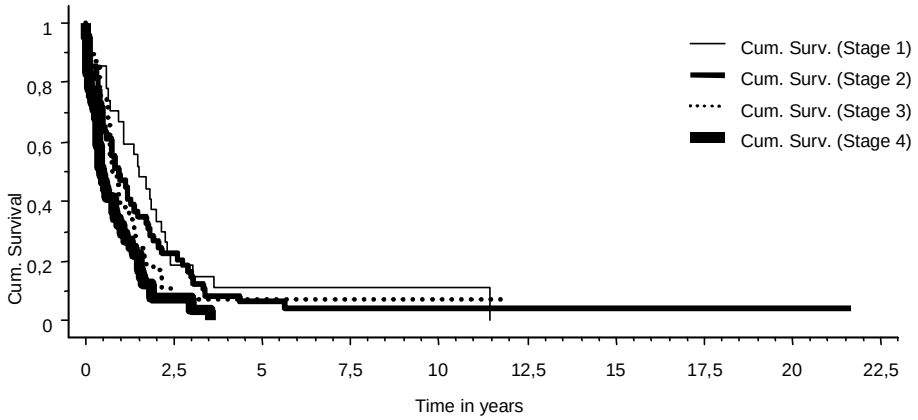


Figure 3 Life tables for Stage 1-4 patients with pancreatic adenocarcinoma. The difference in survival was significant between stages. p=0.0015

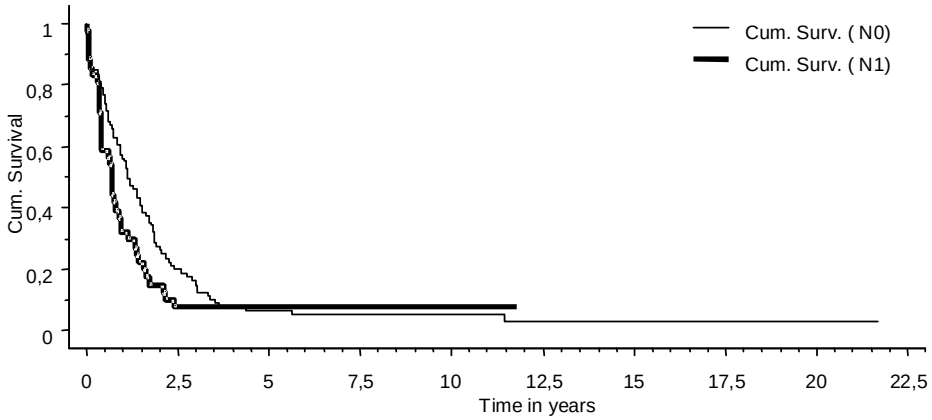


Figure 4 Life tables for node negative (N0) and positive (N1) patients with pancreatic adenocarcinoma. The difference in survival between N0 and N1 patients approached significance p=0.061.

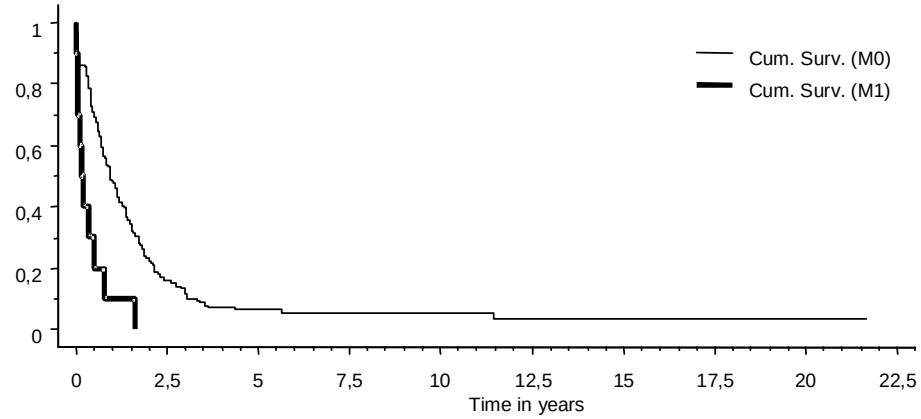


Figure 5. Life tables for patients with local disease (M0) and distant metastases (M1). The difference in survival between M0 and M1 patients is significant, p=0.0001.

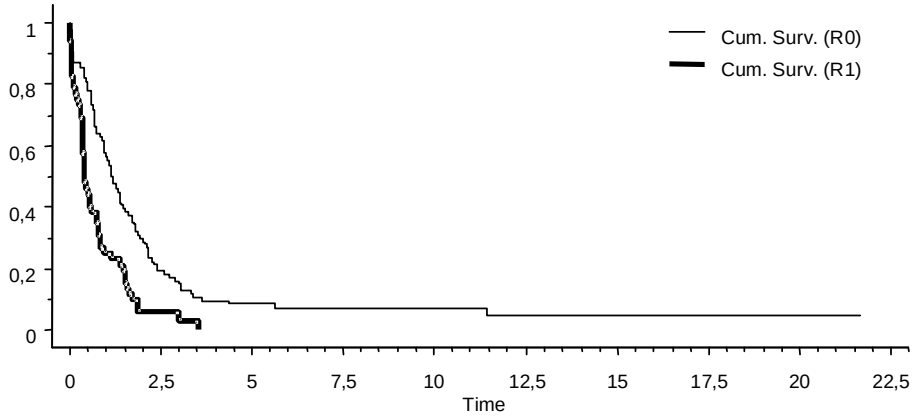
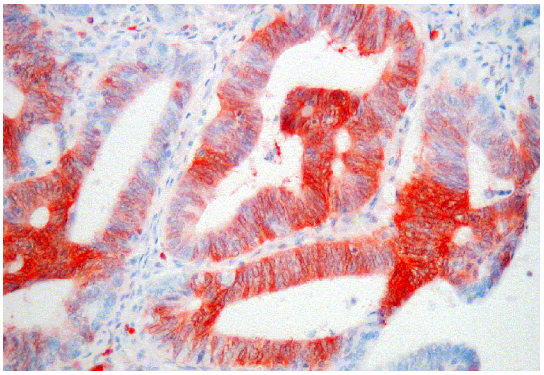
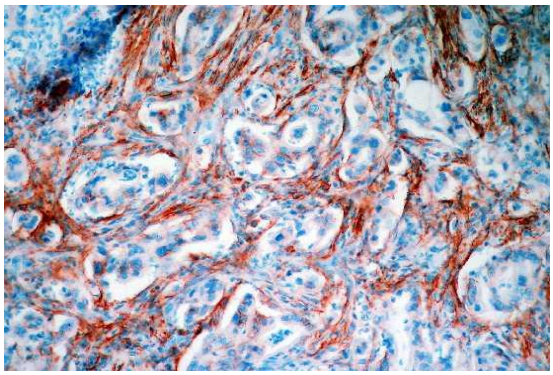


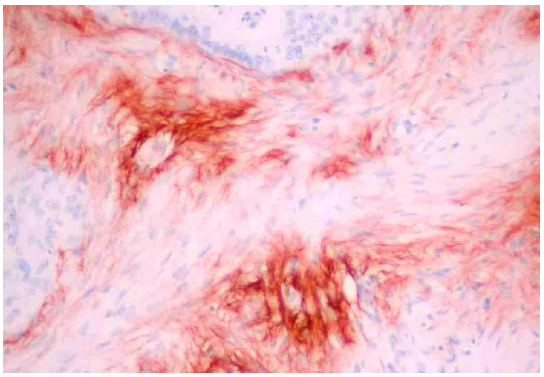
Figure 6. Life tables for radically (R0) and non-radically operated patients (R1). The difference in survival between R0 and R1 patients is significant p<0.0001.



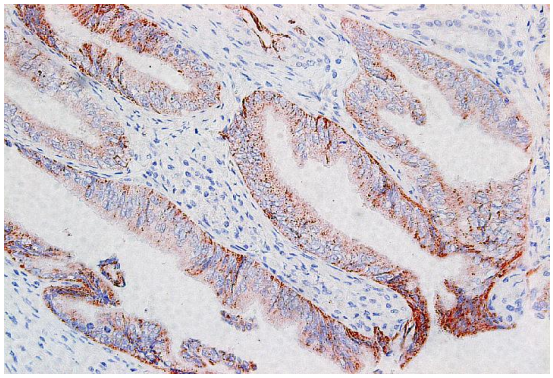
A.



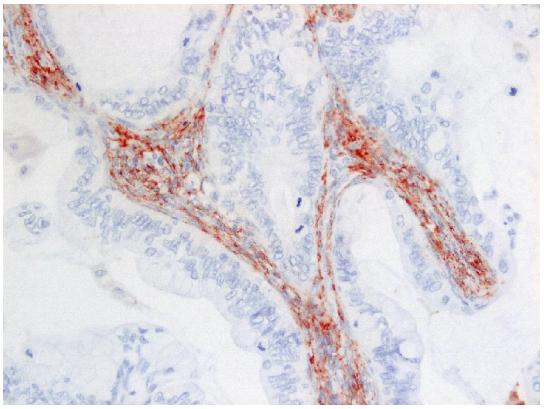
B.



C.



D.



E.

Figure 7. Immunohistochemical staining of A: p27, B: syndecan-1, C: tenascin-C, D: COX-2 and E: MMP 2 in ductal adenocarcinoma of pancreas.

STUDY I (P27)

The expression of p27 was absent or weak in the majority of the pancreatic cancer samples. There were 103 samples with no staining or less than 5% p27 positive cancer cell nuclei, 26 samples with 5-10% positivity, 13 samples with 10-50% positivity and 5 samples with more than 50% of positivity in cancer cell nuclei (Figure 7A) . In positive samples p27 was observed mainly in the nuclei of the ductal cells. When the expression was strong, some staining positivity was also observed in the cytoplasm of the tumour cells. Langerhans islets and lymphocytes expressed p27 also in samples with p27 negative tumours.

The expression of p27was evaluated in 6 samples from acute pancreatitis and 10 from chronic pancreatitis. The expression of p27 was highest in acute pancreatitis: 5 out of 6 (83%) samples were p27 positive and the staining was strong. In chronic pancreatitis the expression was weaker than in acute pancreatitis but higher than in pancreatic cancer. In chronic pancreatitis there were 2 negative, 1 weak, 5 intermediate and 2 strong stainings.

There was no significant correlation between p27 expression and clinicopathological variables such as clinical stage, tumour size, nodal status, metastasis, age or gender. There was a tendency for correlation between loss of p27 and histological grade and tumour location. In univariate analysis surgery for cure ($p<0.0001$), high histological differentiation ($p<0.0001$), non-metastatic disease ($p=0.0001$), less advanced clinical stage ($p=0.002$), tumour location ($p=0.034$) and younger age ($p=0.049$) correlated with longer survival. The association between survival and nodal status approached significance ($p=0.062$). Gender and tumour size were not significantly associated with survival. In stage I-II ($n=76$) the five year survival for p27 negative ($n=56$) patients was 3.6% and for p27 positive ($n=20$) patients 20% ($p=0.03$). In a multivariate survival analysis of the whole patient group ($n=147$), the curability and the tumour grade were independent prognostic factors whereas p27, stage, age and tumour location were not significant factors.

When curabilty, which is highly correlated with tumour stage, was excluded from the Cox regression model, tumour stage was retained as a significant factor in the model. The prognostic significance of p27, along with the other variables potentially related to survival in univariate analysis, was further analysed in stage I-II ($n=76$) in a separate Cox regression model. Grade and p27 were independent prognostic factors, but age, and tumour location did not reach significance.

STUDY II (SYNDECAN-1)

Epithelial immunoreactivity

Syndecan-1 was abundantly expressed on the entire cell surface of ductal cancer cells. In chronic pancreatitis the epithelial staining was observed on the basolateral surface of ductal cells, whereas the acinar cell surface was negative. The epithelial expression of syndecan-1 was slightly enhanced in pancreatic cancer tissue when compared to chronic pancreatitis and strongly enhanced when compared to acute pancreatitis. Syndecan-1 epithelial immunoreactivity was observed in 136 of 144 tumours (94%). The staining result was strong in 18 tumours (12%), moderate in 48 tumours (33%), weak in 70 tumours (49%) and negative in 8 tumours (6%). In chronic pancreatitis the epithelium stained for syndecan-1 in 9 out of 10 (90%) samples. In acute pancreatitis only 2 out of 8 (25%) specimen had weak epithelial expression.

In pancreatic cancer there was no significant association between the level of epithelial syndecan-1 expression and age, gender, grade, TNM stage, tumour size, nodal status, distant metastases or tumour location.

In univariate survival analysis, patients with a lack of epithelial syndecan-1 expression (n=8) had distinctly better survival than patients with epithelial syndecan-1 expression. The number of patients that do not express epithelial syndecan-1 is small (n=8).

When a 20% cut off was used, the epithelial syndecan-1 expression predicted better prognosis in stage I-II, but not in stage III-IV. The 1-, 2- and 5-year survival rates for stage I-II patients with moderate or strong epithelial expression of syndecan-1 (n=33) was 64% (CI 47-80), 42% (CI 26-59) and 15% (CI 3-27) respectively, whereas that of the patients with negative or weak epithelial syndecan-1 expression (n=42) was 45% (CI 30-60), 19% (CI 7-31) and 2% (CI 0-7) respectively (p=0.02). When patients were divided into groups who underwent surgery for cure (n=94) or who received palliative treatment (n=50), the epithelial syndecan-1 was a significant prognostic factor in radically operated patient group, but it was not in the palliativelt treated patient group.

Stromal immunoreactivity

The immunostaining of stromal elements adjacent to cancer cells was usually weak when compared to the epithelial staining (Figure 7B). The stroma was negative in 78 tumours (54%). The immunoreactivity was strong in 11 tumours (8%), moderate in 31 tumours (22%) and weak in 24 tumours (17%). In acute pancreatitis there was no stromal staining for syndecan-1 and only one of ten specimens of chronic pancreatitis showed stromal positivity.

There was no statistically significant association between the level of stromal syndecan-1 expression and age, gender, TNM stage, tumour size, nodal status or distant metastases. The stromal expression was associated with histological grade (p=0.04) and tumour location (p=0.04). The proportion of positive stainings was 14% in grade 1, 48% in grade 2 and 50% in grade 3 tumours. Stromal positivity

was seen in 43% of the tumours located in the head of the pancreas compared to 80% of the tumours in other locations.

In univariate survival analysis stromal syndecan-1 was a significant prognostic factor (p=0.006). The 1-, 2- and 5-year survival rates for patients with stromal syndecan-1 negative tumours (n=78) were 52%, 31% and 8% respectively, whereas the survival rates of patients with stromal syndecan-1 positive tumours (n=66) were 36%, 9% and 3% respectively.

Survival analysis

In univariate survival analysis a lower grade of differentiation ($p<0.0001$) and noncurative resection ($p<0.0001$), stromal syndecan-1 expression (p=0.006), advanced clinical stage (p=0.002), metastasised disease (p=0.0001) and tumour location in the body or tail of the pancreas (p=0.04) correlated with shorter survival. Lymph node status and age reached borderline prognostic significance (p=0.08 and 0.06). Gender and tumour size were not significantly associated with survival. The stromal and epithelial expression was also analysed in combination. Within the group of stromal negative patients, the patients were divided into two groups according to epithelial syndecan-1 expression; Stromal negative/epithelial positive patients had distinctly better outcome compared to patients with negative/negative staining pattern. Within the stromal positive group the epithelial expression did not associate with longer survival. The result was true for the whole patient group (n=144) and in radically operated patients (n=94).

Stromal syndecan-1 was entered into a multivariate Cox regression model with the other factors that were significantly associated with survival ($p<0.05$) in univariate analysis. Three patients with missing data on stage were excluded from the analysis and the total number of patients in the multivariate model was 141.

In Cox regression model only 3 factors retained prognostic significance: possibility of curative resection ($p<0.0001$), grade (grade 1-2 vs. 3) (p=0.0002) and stromal syndecan-1 (p=0.002). Thus, when curability was included in the model, TNM stage (I-II versus III-IV), tumour location and age did not add significant prognostic information. When curability, which is highly correlated with tumour stage, was excluded from the Cox regression model, tumour stage was retained as a significant factor.

STUDY III (TENASCIN C)

A majority of the pancreatic cancer samples expressed tenascin C. There were 12 samples with $\leq$ 5% tenascin positive area, 72 samples with >5 to 20% positivity, 52 samples with >20-60% positivity and 10 samples with more than 60% positivity in the stroma. Tenascin C was observed mainly in the stroma surrounding malignant ductal cells. When the expression was strong, the staining was seen as intense undulating bands around tumour cells in the adjacent stroma (Figure 7C).

The staining pattern of tenascin C in chronic pancreatitis samples was similar to that in pancreatic cancer, positivity for tenascin C was seen diffusely in the stroma surrounding ductal cells. In acute pancreatitis there was a distinct pattern of tenascin C expression: tenascin C was expressed as bands between lymphatic cells at the edges of remaining pancreatic tissue, bordering necrotic areas. The expression of tenascin C was stronger in acute pancreatitis than in chronic pancreatitis. In acute pancreatitis there were 0 negative, 4 weak, 3 intermediate and 3 strongly stained samples. In chronic pancreatitis there were 4 negative, 1 weak, 4 intermediate and 1 strongly stained sample. In normal pancreatic samples there was no tenascin expression in the pancreatic tissue. In one section there was some positivity surrounding a large bile duct.

Tenascin C expression correlated with low differentiation (grade 1-2 vs. 3) and with age ($\leq$66 years vs. >66 years). In grade 1 and 2 tumours 37% of the samples had an intermediate or strong tenascin C expression whereas in grade 3 tumours 56% of the samples had an intermediate or strong tenascin C expression (p=0.038). Younger male patients had a stronger tenascin C expression in the tumours compared to tumours of the older male patients or all females. In male patients under 66 years (n=35) 66% of the tumours had an intermediate or strong tenascin C expression, whereas in male patients aged over 66 years (n=30) only 27% of the tumours had an intermediate or strong tenascin C expression. There was no significant correlation between tenascin C expression and clinical stage, tumour size, nodal status, distant metastasis, tumour location or gender.

In univariate analysis, surgery for cure (p<0.0001), high histological differentiation (p=<0.0001), absence of distant metastases (p=0.0001), less advanced stage (p=0.002), tumour location in the head of the pancreas (p=0.04) and young age (p=0.03) correlated with a longer survival. The association between survival and nodal status (N), tumour stage (T) and tumour size approached significance (p=0.07). Tenascin C did not associate with survival in the entire patient group (Figure 2a in study III). In the subgroup of male patients (n=65) tenascin C expression (>20%) associated with longer survival (p=0.038, $\chi^2 = 4.3$). Male patients with $\leq$ 20% of tenascin C expression had 29% (CI 14-45%), 15% (CI 3-27%) and 0% (CI 0 – 0%) 1-, 2- and 5-year survival, respectively. Male patients with > 20% tenascin C expression had 58% (CI 41-75%), 26% (CI 10-41%) and 11% 1-, 2- and 5 year survival, respectively. In other subgroups, divided by gender, age, tumour location, stage or tumour location, tenascin C expression did not associate to survival (data not shown).

STUDY IV (COX-2)

Immunoreactivity of COX-2 was evaluated in 128 pancreatic ductal adenocarcinomas of which 82 (64%) were negative and 16 (13%) weakly, 27 (21%) moderately and 3 (2%) strongly positive. COX-2 expression was seen in cytoplasmic granules of ductal tumour cells, whereas the stroma was negative (Figure 7D). Islet cells stained positive in all samples, also in those with no COX-2 expression in the tumour.

There was no correlation between COX-2 expression and gender, histological grade, tumour stage, tumour stage, nodal status, tumour size (≤2cm vs. 2-4cm vs. >4cm), curativity or tumour location (caput vs. other location). COX-2 expression was associated to distant metastases (n=9); there was no COX-2 expression in any of the primary tumours with distant metastases (p=0.026). COX-2 was expressed more frequently in samples of older patients (>62 years) although the difference was not statistically significant (p=0.0655) (Table 1 in study IV).

The survival among patients with COX-2 negative tumours was significantly (p=0.011) better than among patients with COX-2 positive tumours. One-, two- and five-year survival rates were 34, 5 and 5% in COX-2 positive categories, compared to 51, 32 and 8% in the COX-2 negative category, respectively. The median survival for patients with COX-2 positive tumours was 8.1 months, compared with 13.2 months for patients with COX-2 negative tumours. Low histological grade, low TNM stage, no distant metastases and curativity showed strong association with better survival in univariate survival analysis (p<0.001). Young age (p=0.042), low tumour stage (p=0.045), small tumour size (p=0.045) and tumour location in the pancreatic head (0.045) were also associated to better prognosis.

Within the group of radically operated patients COX-2 expression correlated with survival in univariate analysis (p=0.004). One-, two- and five-year survival rates were 40, 7 and 0% in COX-2 positive categories, compared to 67, 46 and 11% in the COX-2 negative category, respectively. Median survival was 10 months for COX-2 positive patients, compared to 20 months for patients with COX-2 negative tumour.

In multivariate analysis COX-2 retained its independent prognostic significance (p= 0.018). TNM stage and histological grade (HR 3.5) were the strongest independent prognostic factors followed by COX-2 (HR 1.6).

STUDY V (MMP-2)

Epithelial immunoreactivity

MMP2 was abundantly expressed at the invasive front of the tumour. In tumour cells MMP-2 expression was seen in the nuclei and cytoplasm. MMP-2 epithelial immunoreactivity was observed in 64 of 127 tumours (50%). The staining result was strong in 6 tumours (5%), moderate in 26 (20%), weak in 32 (25%) and negative in 63 tumours (50%).

MMP-2 expression was associated with higher TNM Stage (p=0.0016). Thirteen percent of stage I and II tumours were MMP-2 positive (>10% of cells), whereas in stage III 25% of tumours and in stage IV 49% of tumours were MMP-2 positive (>10% of cells). Also tumour stage (T) and distant metastasis (M) were associated to MMP-2 expression (p=0.0078 and 0.0032, respectively). Nodal metastasis (N) had a tendency to association to MMP-2 (p=0.0857). Non-curativity was also associated to MMP-2

expression; 15 % of tumours that were resectable had MMP-2 positivity compared to 44% of tumours that were not curatively resectable (p=0.0004). There was no statistically significant association between the level of epithelial MMP-2 expression and age, gender or grade.

In Kaplan-Meier univariate survival analysis, patients with epithelial MMP-2 negative (10% cut-off) tumours (n=95) had better survival than patients with epithelial MMP-2 positive tumours (n=32, p=0.035) (Figure 2 in study V). The 1-, 2- and 5-year survival rates for patients with epithelial MMP-2 negative tumours was 49% (CI 39-59), 26% (CI 17-34) and 8% (CI 2-13) respectively and for patients with MMP-2 positive tumours (n=32) 31% (CI 15-47), 9% (CI 0-20) and 6% (CI 0-15) respectively (p=0.035). When the analysis was done in four categories i.e. MMP-2 $\leq$ 5%, MMP-2 5-10%, MMP-2 10-50% and MMP-2 >50% there was no statistical difference. In multivariate survival analysis MMP-2 was not an independent prognostic factor.

Stromal immunoreactivity

There was stromal MMP-2 expression in 107 tumours (84%). The immunoreactivity was moderate in 18 tumours (14%) and weak in 89 tumours (70%) (Figure 7E). Stromal MMP-2 expression (positive vs. negative) had no correlation to age ($\leq$62 vs. >62), gender, grade (I vs. II vs. III), TNM stage (I, II, III vs. IV), tumour size ($\leq$2cm vs. 2-4cm vs.>4cm) or distant metastases (M0 vs. M1). The stromal expression was associated with nodal metastasis (p=0.03). The proportion of positive stainings was 11% in node negative tumours and 26% in node positive tumours, p=0.03. In univariate survival analysis stromal MMP-2 was not associated with survival (p=0.859).

PROGNOSTIC SIGNIFICANCE OF P27, STROMAL SYNDECAN-1, COX-2 AND EPITHELIAL MMP-2

In univariate analysis TNM stage, grade, age, stromal syndecan-1, COX-2 and epithelial MMP-2 had prognostic significance, when analysed in the whole patient group (Table 9 and Figure 8). When all covariants were entered to multivariate analysis, grade, stage, age and stromal syndecan-1 were independent prognostic factors, but COX-2 and MMP2 were not.

Table 9 Prognostic significance of the series of tumour markers (Study I-V) in univariate analysis.

	n	Cut-off value (%)	Median survival (months)	1-year survival (%)	2-year survival (%)	5-year survival (%)	p-value
p27 (in stage I and II)	76		13				
	56	≤5	12	50	25	3.6	0.03
	20	>5	22	65	40	20	
Syndecan-1	144		10				
Stromal	78	≤5	13	52	31	8	0.006
	66	>5	8	36	9	3	
Epithelial	78	≤20	9	42	17	3	0.2
	66	>20	11	48	26	9	
Tenascin C	146		10				
	84	≤20	10	44	21	5	0.34
	62	>20	10	47	21	7	
COX-2	128		10				
	82	≤5	13	51	32	8	0.011
	46	>5	8	34	5	5	
MMP 2	127		10				
Stromal	109	≤10	10	45	23	7	0.79
	18	>10	10	42	12	6	
Epithelial	95	≤10	11	49	26	8	0.04
	32	>10	7	31	9	6	

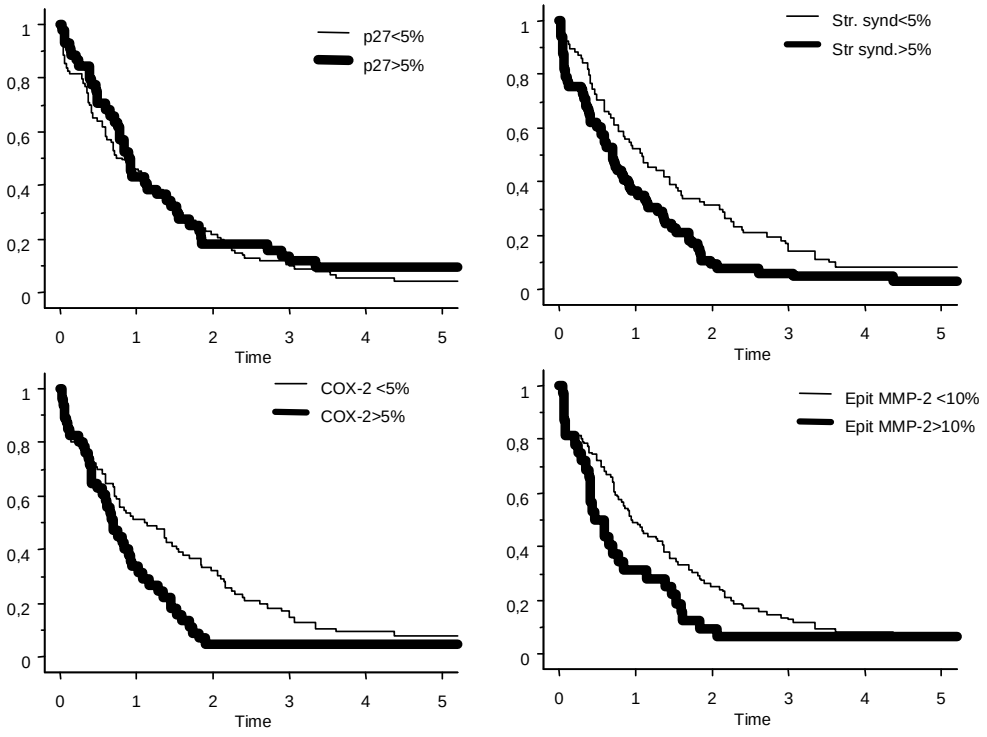


Figure 8 Kaplan-Meier survival curves for patients according to the tumour expression of p27, stromal syndecan-2, COX-2 and epithelial MMP-2 in the whole patient group .

9. DISCUSSION

TNM STAGING

All tumours were re-staged in 2000-2001 using the fifth edition of the UICC TNM Classification, which was the latest at the time of the study. In 2002 the sixth edition was released and the criteria of staging of pancreatic exocrine tumours were modified again. In order to keep all publications uniform and comparable, patient material was not re-staged again after 2002. To stage the tumours as accurately as possible retrospectively, operation reports and pathology records were carefully studied. In some cases the microscopy slides revealed tumour growth to be more extensive than determined in the records. In the material of 147 patients operated for pancreatic cancer the TNM stage was an independent prognostic factor in univariate analysis ($p=0.002$) as well as in multivariate analysis ($p=0.01$) as shown in Table 3 in study I. Stage is highly associated with curability, either curability or stage was used in the multivariate analysis as a covariant. Nodal status only approached significance in univariate analysis ($p=0.06$). This shows the difficulties of determining nodal status from archival samples. Nodal dissection was not as extensive at the time those patients were operated as it is today. Many node positive patients were apparently staged as node negative since the node metastasis were not detected. Therefore, TNM staging is not a perfect way to determine patients' prognosis and clearly more feasible and accurate tumour markers are needed.

TIME OF OPERATION AND EFFECT ON SURVIVAL

Patients were operated on between the years 1974 to 1998. When patients operated on in the 1970´s were compared to patients operated on during the 1980´s and 1990, there was a significant improvement in patient survival. One-year survival rates of radically operated patients in the 1970´s, 1980's and 1990's were 47, 53 and 66%, respectively, while the 5-year survival rates were 0, 10, and 12%, respectively. This reflects that the treatment of pancreatic cancer patients has changed over the decades in many of the specialised centres. Firstly, the pre-and postoperative care has improved due to advancements in anaesthesiology. Secondly, pancreatic operations are centralised to specialists in pancreatology with the additional benefits of extended lymphadenectomy introduced at the end of nineties. Also the operation mortality has decreased dramatically over the decades. Patients undergoing pancreaticoduodenectomy for cure in the 1970´s and 1980´s had a post-operative mortality of 16% and

13%, respectively, yet in the 1990´s it was as low as 2.8%. Even though the results of surgery have improved over the decades, it should not have any effect on different tumour markers and their significance on survival. To confirm this, post-operative mortality patients were included, excluded or cencored and showed that this did not influence the results as shown in study I to V.

PATIENT MATERIAL, TUMOUR DIAGNOSIS AND HISTOLOGICAL GRADE

In addition to ductal adenocarcinoma, the most common form of pancreatic cancer, there are several less common subtypes many of which have a better prognosis than ductal adenocarcinoma. However, it is true that presenting careful histopathological review of all tumours is mandatory to achieve reliable survival data (Alanen et al. 1993; Carpelan-Holmström et al. 2005). In order to exclude all misdiagnosis, one pathologist carefully studied each tumour and evaluated the diagnosis and grade of differentiation. Several patients with other diagnoses were excluded such as islet cell carcinoma, cystadenocarcinoma, carcinoma of papilla Vateri and lymphoma. There were 216 patients with histologically verified pancreatic adenocarcinoma. One patient was excluded from the study due to missing patient records, 48 due to missing histological specimens and 20 due to inadequate samples. Thereafter, 147 patients were available with adequate sample for immunohistological evaluation. The 20 patients with inadequate samples were mostly palliatively treated patients with only a small biopsy or fine-needle sample available and not acceptable for immunohistochemical evaluation. The 48 random patients with missing sample did not differ in other terms of clinicopathological variables compared to those patients included. In the study group the overall and disease specific, 5-year survival rates were 4.8 % and 5.8% and for radically operated patients 7.4 and 9%. Six patients that were alive at the end of study and 2 patients who died from other causes were censored. These results are comparable to other studies in Europe at that time period as shown in table 10.

Table 10 Survival of pancreatic ductal adenocarcinoma after radical pancreaticoduodenectomy in different studies.

First author and year	N	Source	Treatment years	5-year survival
Kuhlmann 2004	160	Amsterdam, Netherland*	1992-2002	8 %
Wagner 2004	160	Bern, Sveitsi*	1993-2001	24 %
Cleary 2004	123	Toronto, Canada	1988-1996	15 %
Capussotti 2003	100	Candiolo, Italia*	1988-1998	8 %
Yeo 1998	149	Maryland, USA*	1970-1992	15%**
Yeo 1995	143	Maryland, USA*	1970-1991	26 %
Bramhall 1995	208	West Midland, UK	1957-1976	2.6%
	145		1977-1986	9.7%
Nitecki 1995	174	Mayo Clinic, USA*	1981-1991	7 %

*single clinic study, ** periampullary, pancreatic primary adenocarcinoma

Histiological grade of differentiation has been a significant prognostic factor in most studies (Luttges et al. 2000), although it is highly dependent on the pathologist. When original grades, assessed by different pathologists were analysed, the association between grade and survival only approached significance (p=0.05). In this study, the grade of differentiation was re-evaluated by a single pathologist, blinded to patient outcome. Grade of differentiation had the best significance of all tumour related clinicopathologic factors in univariate ($p<0.0001$) and in multivariate analysis (HR= 3.6, p=0.0002), as shown in table 2 and 3 in study I.

TUMOUR MARKERS IN THE STUDY

Potentially useful markers were chosen for the study using three criteria. Firstly, the gene or protein had to play an important role in tumour pathogenesis. Secondly, it was a potential prognostic factor in other carcinomas, preferably in ductal adenocarcinomas. Thirdly, it had not been previously studied in large series of pancreatic cancer and had not shown to be a prognostic factor in multivariate analysis.

p27

p27 is a CDK-inhibitor and prevents progression of the cell cycle from G1 to S phase (Sherr et al. 1995). Loss of p27 expression in tumours has been shown to be associated with poor prognosis in breast, lung, prostate and bladder cancer (Esposito et al. 1997; Porter et al. 1997; Yang et al. 1998; Del Pizzo et al. 1999). In one study with a relatively small patient number (n=35) it was shown that p27 was associated with poor outcome in patients operated on for pancreatic cancer (Lu et al. 1999). It was necessary to confirm this result with a larger number of patients. p27 is present in nuclei of cells and expressed in normal healthy tissue. In pancreatic cancer expression of p27 is down-regulated. In the current study p27 was expressed in 30% of the pancreatic ductal adenocarcinoma samples. In benign samples of pancreatitis, p27 was expressed in 13 of 16 samples (81%). In the whole cancer patient group p27 was not a prognostic factor. However, in a series of patients operated on for cure (n=95) or stage I and II patients (n=76) lack of p27 expression was associated with shorter survival and this was true also in multivariate analysis. Similar results have also been recently reported (Feakins et al. 2003; Fukumoto et al. 2004). The loss of p27 expression probably reflects the aggressive nature of the disease. Furthermore, up-regulation of p27 by pharmacologic inhibition of RAF to MEK to ERK signalling pathway has been proposed as a novel target for chemotherapy (Gysin et al. 2005).

Syndecan-1

Syndecan-1 is a transmembrane receptor that participates in cell-cell and cell-matrix interactions, as well as in cell proliferation. Syndecan-1 is a receptor to various extracellular matrix proteins such as interstitial collagen, tenascin, fibronectin and basic fibroblast growth factor (Koda et al. 1985; Saunders et al. 1988; Salmivirta et al. 1991; Salmivirta et al. 1992). The type and structure of syndecans may vary in different cell types, resulting in cell type specific binding capacity (Sanderson et al. 1994). In

pancreatic cancer expression of syndecan-1 is upregulated, but in many other cancers it is downregulated (Conejo et al. 2000). Syndecan-1 has been associated with shorter over-all and recurrence free survival in other cancers but not in pancreatic cancer (Inki et al. 1994; Anttonen et al. 1999). In study II, stromal syndecan-1 was both upregulated and associated with worse outcome (n=144, p=0.002). In benign samples there was no syndecan-1 in pancreatic stroma except in one chronic pancreatitis sample. Stromal syndecan-1 in cancer may bind to ECM molecules and thereby participate in cell migration and enhance tumour growth and migration (Woods 2001). Epithelial syndecan is thought to maintain cell morphology. In the current study it was shown that in benign samples the epithelial syndecan-1 was localised on the basolateral cell surface, but in cancer cells there was no polarisation present and syndecan-1 was present around the whole cell membrane. Loss of epithelial expression of syndecan-1 was associated with worse outcome in univariate analysis in the subgroup of patients who underwent surgery for cure (n=94, p=0.03). Study II was the first to report associations between stromal and epithelial syndecan-1 and pancreatic cancer patients' outcome. It has been demonstrated that soluble syndecan-1 in serum correlates to worse outcome in multiple myeloma and non-small cell lung cancer (Joensuu et al. 2002; Lovell et al. 2005). It remains to be shown whether soluble syndecan-1 in serum is a prognostic marker also in pancreatic cancer.

Tenascin C

Tenascin C is a large spider-like molecule and a member of the adhesion modulating family of ECM proteins. Tenascin C is expressed transiently during fetal development and in areas of growing or differentiating epithelia in adult tissue as well as in the stroma of a variety of tumours (Erickson et al. 1989; Koukoulis et al. 1991). Tenascin C expression has been associated with tumour recurrence and prognosis, although results have been contradictory (Vaidya et al. 1996; Kressner et al. 1997; Jahkola et al. 1998; Pilch et al. 1999; Juhász et al. 2000; Emoto et al. 2001; Atula et al. 2003; Wiksten et al. 2003). From the literature, there were no studies on pancreatic cancer outcome and tenascin C. Study III shows that tenascin C is expressed in most samples of pancreatic cancer (92%). High tenascin C expression (>20%) correlated with younger age ($\leq$66 years) and poor grade of differentiation (G1 and 2 vs. G3). Tenascin C did not correlate with outcome of patients. There was no expression of tenascin C in normal pancreas, but in pancreatic cancer expression was abundant. Moreover, expression correlated with differentiation. These findings are in accordance with the anti-adhesive functions of tenascin C as described previously (Wenk et al. 2000; Midwood et al. 2002). In the current study it was shown that in inflammation of pancreas, both in acute and chronic pancreatitis, ductal cells expressed tenascin C.

COX-2

The association between inflammation and cancer was already established in the 19th century by Rudolf Wirchow (Balkwill et al. 2001). Both hereditary and sporadic chronic pancreatitis are risk factors for pancreatic cancer (Lowenfels et al. 1993; Bansal et al. 1995; Lowenfels et al. 1997). In chronic pancreatitis there is a strong desmoplastic reaction around ductal cells that is also seen in pancreatic cancer. Sromal cells produce cytokines, growth factors, and inflammation mediators that are known to

induce COX-2 expression (McCawley et al. 2001). COX-2 is over expressed in chronic pancreatitis as it is in pancreatic cancer (Tucker et al. 1999; Schlosser et al. 2002). In tumourigenesis, COX-2 has been proposed to take part in stimulation of proliferation, in inhibition of apoptosis, and in invasion by enhancing production of MMPs and by promoting angiogenesis (DuBois et al. 1996; Tsujii et al. 1997; Tsujii et al. 1998). Since COX-2 is inducible and implicated in epithelial tumour development, it may be hypothesised that COX-2 expression in pancreatic cancer would lead to poor prognosis. Furthermore, elevated COX-2 expression is associated with poor outcome in several other cancer forms such as colon, breast, gastric and ovarian cancers (Sheehan et al. 1999; Ristimäki et al. 2002; Shi et al. 2003; Erkinheimo et al. 2004). In the current study COX-2 was expressed in 36% of the pancreatic cancer samples. Elevated COX-2 expression was associated with poor prognosis and it was independent of stage and grade (RR 1.6). Kokawa et al. showed that COX-2 correlated with the inhibition of cell growth by aspirin in four pancreatic cancer cell lines and proposed chemoprevention by COX inhibitors (Kokawa et al. 2001). Also other groups have demonstrated tumour growth inhibition by COX-2 inhibitors, but this was COX-2 independent expression (Molina et al. 1999; Eibl et al. 2003). In preclinical studies, COX-2 inhibitors enhanced the antitumoural efficacy of gemcitabine (Yip-Schneider et al. 2001).

Earlier studies and the results presented in this thesis on the prognostic significance of COX-2 support efforts to initiate clinical trials to evaluate whether tumour expression of COX-2 could be utilized to define patients who will benefit from neoadjuvant therapy.

MMP-2

Degradation of ECM by different proteases is an essential step in tumour cell penetration and infiltration into adjacent tissues and vessels and thereby to distant organs (Keleg et al. 2003). MMP-2 (gelatinase A) belongs to a family of matrix metalloproteinases that catalyses ECM degradation (Kleiner et al. 1999). MMP-2 degrades type IV collagen, the major component of epithelial basement membranes and renders tumour cells more invasive (Birkedal-Hansen 1995). MMPs are upregulated and related to disease recurrence and poor survival in ovarian, gastric and breast cancer (Allgayer et al. 1998; Talvensaari-Mattila et al. 2003; Torng et al. 2004). Normal pancreas does not express MMP-2, but in pancreatitis and cancer MMP-2 is upregulated (Bramhall et al. 1997). Prognostic significance of MMP-2 is yet to be fully elucidated (Kuniyasu et al. 1999; Yamamoto et al. 2001; Bloomston et al. 2002). In the current study MMP-2 expression was seen in 50% of epithelial cells and in 84% of the stroma. Epithelial MMP-2 expression was associated with advanced tumour stage, tumour status, metastatic disease, curability and in univariate analysis with worse survival. Stromal MMP-2 expression did not correlate with clinicopathologic factors. Thesis results are in accordance with the biological role of MMP-2 in enhancing tumour invasion and metastasis.

Importance of tumour markers

Treatment of pancreatic cancer by surgery has improved significantly over the years: surgery is centralised in many clinics and postoperative mortality is below 3%. Although survival has somewhat

improved, surgery is not the answer for additional advances in treatment. Chemotherapy will probably bring the greatest improvements in the treatment of pancreatic cancer. In order to find a suitable oncological treatment to inhibit and prevent tumour cell progression and invasion, the biological behaviour of pancreatic cancer needs to be studied. Genetic and molecular changes in tumours could reveal new diagnostic and therapeutic target molecules. Although tissue markers studied i.e. p27, syndecan-1, COX-2 and MMP2 seem to have prognostic significance in pancreatic ductal adenocarsinoma, they have limited value in clinical practice. However, these molecules may have use as targets for chemotherapy. In addition, tumour markers may have use in selecting those patients who benefit from chemotherapy.

10. CONCLUSIONS

- Tissue expression of p27 is a significant predictor of 5-year survival in stage I-II pancreatic adenocarcinoma

- Stromal syndecan-1 expression is an independent prognostic marker in pancreatic cancer, whereas epithelial syndecan-1 expression predicts better prognosis only in resectable disease.

- Tenascin C expression is increased in most pancreatic carcinomas, but contrary to the results in other cancers, it is not a prognostic factor in pancreatic cancer. Tenascin C is associated with grade of differentiation.

- The expression of COX-2 was associated with poor outcome and it was independent of tumour stage, grade and age in multivariate analysis.

- Epithelial MMP-2 expression correlates with tumour stage and is associated with poor survival in pancreatic adenocarcinoma.

11. ACKNOWLEDGEMENTS

This work was carried out at the Department of Surgery, University of Helsinki between the years 2000 and 2005. I am sincerely thankful to everyone who has encouraged and supported me on this work over the years.

I express my respect and appreciation to Professor Eero Kivilaakso for providing me excellent facilities at the Department of Surgery.

I am deeply grateful to supervisor Docent Caj Haglund, who introduced me to the interesting world of research, which I had previously slightly ignored. He has facilitated first-class working places at Terkko and grants, thereby providing a perfect setting for research. His endless enthusiasm, inspiring ideas and optimism have encouraged me through this project.

I want to acknowledge Professor Timo Paavonen and Docent Ilmo Kellokumpu, reviewers of this thesis, for their prompt and professional work. I want to thank Ilmo for constructive comments and new ideas throughout the book.

I am also deeply thankful for Docent Tuula Kiviluoto for giving me valuable comments on clinical aspects of pancreatic cancer. As my tutor in gastric surgery she has taught me practical pancreatology, which I highly appreciate. I consider myself fortunate to have had an opportunity to work at her clinic of expertise on pancreatic cancer.

I want to express my appreciation to all my co-authors, Stig Nordling, Johan Lundin, Ari Ristimäki, Johanna Louhimo and Kristina von Boguslawski. I owe my deepest thanks to Docent Stig Nordling for guidance to pathology of pancreatic cancer. He has inspected thousand of slides of pancreatic cancer for this work with incredible expertise, accuracy and speed. He has given me realistic opinions of my work by deep voice of experienced pathology. I wish to thank Johan Lundin for teaching me statistics and for his statistical analysis of my work. He has also had great impact on the manuscripts by giving me expertise opinions on those. Ari Ristimäki has taught me about COX-2 in cancer and also photographed slides for publication, and I am grateful for that. I want to give heartful thanks to Johanna Louhimo, who has given me priceless advice on practical research, as well as being a good friend.

The warmest thanks I owe to research secretary Elina Aspiala, who has been there and helped with all minor and major problems. With out her help, the book would (possibly) have got ready, but a lot later! I am also much obligated to laboratory technicians Elina Laitinen and Päivi Peltokangas for professional job with the immunohistochemistry.

Doctor Phil Rye is acknowledged for language editing this book. I was warmly delighted for his proposal to take this challenging task of converting my “Savo”- English to proper and understandable language.

My warmest appreciation goes to my dear friends at “Terkko office”: Johanna Louhimo, Päivi Siironen, Johanna Mrena and Monika Carpelan-Holmström. We have showed the girl surgeon power on different fields: research related trips to Spain, Germany and US, as well as on sportive trips to Stockholm Marathon and Lapland. Conversations with you have made the world so much better place! I also want to thank Päivi and Johanna L, for advising me about practical organization of the dissertation day.

I offer my warm thanks to colleges at Porvoo, Peijas and Meilahti hospitals. I especially want to thank colleges Juha Hahl, Marja Leivonen, Sini-Marja Sjöblom, Kari Mikkola, Ilkka Saario and Marianne Berggren, Olli Suomalainen and Claes Tötterman, as well as other surgeons at Peijas and Porvoo Hospitals for coaching me during the early years of surgical carrier. I also want to thank colleges in Meilahti hospital at the Dapartment of Surgery for gastric surgery education.

The important task of combining gastroenterology and leisure has been perfectly achieved by our members of “Kanalogi yhdistys” Saija Vuolio, Marian Ahlskog-Karhu, Mia Rainio, Suvi Leutola, Carola Haapamäki and Pia Manninen. Our regular meetings around Finland have been cheerful and unforgettable events. I am privileged to have such good college friends as you are!

I want to thank my parents Satu and Markku Juuti for giving me good basis for life. You have encouraged me through and given me faith and self-confidence at times of chaos in-between motherhood, clinician and researcher. I also want to thank my beloved sisters Mari, Helmi and Liisa for helping me with everyday life, especially with my daughter Eevi.

I am forever grateful to my dear husband Kimmo, who has stood by me through these somewhat busy years. I am glad that he has constantly reminded me that there is also other life than surgery and research. I am fortunate to have a husband who shares enthusiasm to our mutual hobbies, such as travelling, marathon and diving. And most importantly, I want to thank him for being such an excellent father for our princess Eevi. I also want to thank my sweetest little girl Eevi, who was born 2 weeks before this project started.

Helsinki, January 2006

Anne Juuti

12. FINNISH SUMMARY (TIIVISTELMÄ)

Tausta: Haiman duktaalinen syöpä on kolmanneksi yleisin mahasuolikanavan pahanlaatuisista kasvaimista paksusuolisyövän ja mahasyövän jälkeen. Vuonna 2003 Suomen syöpärekisterin mukaan uusia haimasyöpään sairastuneita potilaita oli 8,5 miestä ja 7,3 naista 100 000 asukasta kohden. Haimasyöpä on tunnettu huonon ennusteen vuoksi: lähes kaikki potilaat kuolevat viiden vuoden sisällä. Haimasyövän ensioireet ovat vähäiset ja tauti etenee nopeasti. Oireiden ilmaantuessa syöpä on usein jo levinnyt, eikä parantavaa hoitoa voida antaa. Vain joka viides kasvain todetaan varhaisvaiheessa, jolloin se voidaan poistaa leikkauksella. Haimasyövän tyyppileikkaus on pankreatikoduodenektomia, jossa poistetaan haiman pää ja pohjukaissuoli. Leikkaushoitoon voidaan yhdistää taudin kulkua hidastava solunsalpaajahoito. Valitettavasti radikaalikirurgian ja solunsalpaajahoitojen jälkeenkin taudilla on tapana uusia - joskin hoitotulokset ovat selkeästi parantuneet viimeisten 15 vuoden aikana. Haimasyöpään erikoistuneissa keskuksissa 5-vuotis ennuste on kohentunut 5:stä aina 25%:een. Taudin ennusteen määrittämiseksi on perinteisesti ollut käytössä levinneisyysluokitus (TNM-luokitus) ja kasvaimen erilaistumisaste (Grade). Näiden lisäksi kaivataan tarkempia ennustetekijöitä, joiden avulla saataisiin lisätietoa taudin tulevasta kulusta.

Tutkimuksemme tarkoituksena on ollut testata uusia, mutta lupaavia biologisia syövän ennusteellisia merkkiaineita immunohistokemiallisin menetelmin.

Aineistossamme on vuosina 1974 – 1998 Helsingin yliopistollisessa keskussairaalassa leikatut haimasyöpäpotilaat, joiden kasvaimesta on säilynyt kudosnäyte patologian laitoksen arkistoissa. Näytteet tutkittiin uudelleen ja aineistosta poistettiin ne potilaat joilla osoittautui olemaan väärä diagnoosi, kuten haiman kystadenokarsinooma tai insulinooma. Duktaalista haimasyöpää sairastavia potilaita aineistoon soveltui 149.

Menetelmä: Potilastiedot kerättiin sairaskertomuksista, syöpärekisteristä ja väestörekisteristä. Patologi tutki kudosnäytteet, varmisti diagnoosin ja määritti erilaistumisasteen WHO:n kriteerien mukaan. Näytteet värjättiin työryhmän laboratoriossa immunohistokemiallisin menetelmin. Työssämme tutkittiin p27, syndekaani-1, tenaskiini C, syklo-oksygenaasi-2 (COX-2) ja matriksi metalloproteinaasi –2 (MMP-2) esiintyvyys haimasyöpäkasvaimissa ja verrattiin tulosta elossaoloaikaan sekä muihin muuttujiin, kuten kasvaimen erilaistumisasteeseen, kasvaimen kokoon, imusolmukkeisiin levinneisyyteen ja etäpesäkkeisiin.

Tulokset: Ennusteeseen vaikuttavia tekijöitä olivat kasvaimen erilaistumisaste, levinneisyysaste, tuumorin paikallinen levinneisyys, etäpesäkkeet, radikaalileikkaus, kasvaimen sijainti, potilaan ikä,

strooman syndekaani-1, COX-2 ja epiteelin MMP-2. Potilailla, joille oli tehty parantava leikkaus, edellä olevien muuttujien lisäksi ennustetekijöitä olivat p27 ja epiteelin syndecan-1. Itsenäisiä ennustetekijöitä olivat erilaistumisaste, TNM-luokitus ja radikaalileikkaus, strooman syndekaani-2 ja COX-2 sekä radikaalileikatuilla potilailla p27.

Johtopäätökset: Syndekaani-1:n, COX-2:n ja MMP-2:n esiintyvyydet syöpäkudoksessa antavat uutta ja itsenäistä tietoa aiemmin käytettyjen ja tunnettujen ennustetekijöiden lisäksi.

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14. ORIGINAL PUBLICATIONS