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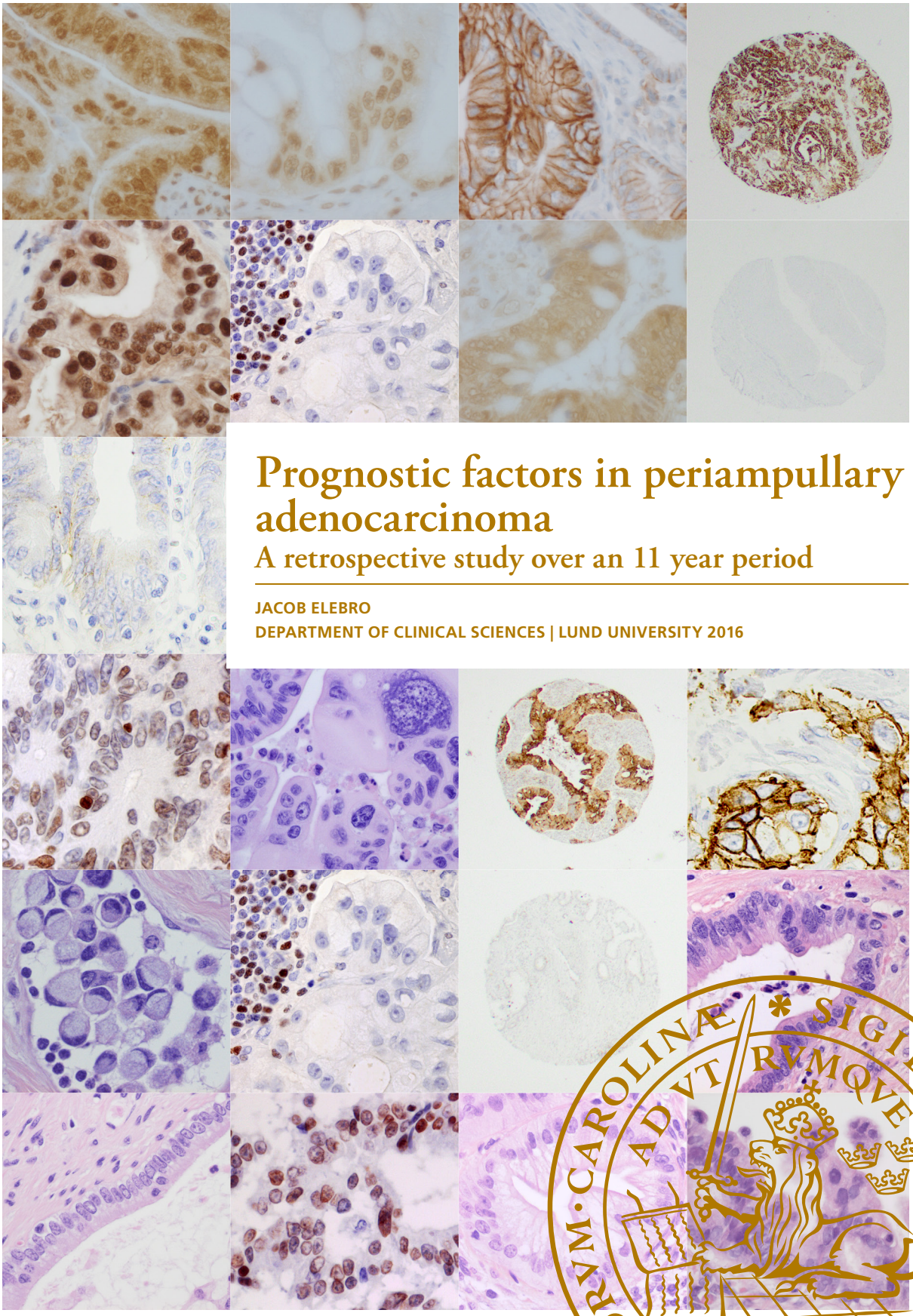
Elebro, Jacob

2016

Document Version:
Publisher's PDF, also known as Version of record



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A retrospective study over an 11 year period

JACOB ELEBRO
DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY 2016

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Jacob Elebro

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden,
to be defended at the Lecture Hall of the Radiotherapy Building, 3rd floor,
Department of Oncology, Skåne University Hospital, Lund
Friday May 13, 2016 at 09.15 am.

Faculty opponent

Professor Ivar Prydz Gladhaug
Head of Department, Institute of Clinical Medicine
University of Oslo, Norway

Organization	Document name
LUND UNIVERSITY	Doctoral dissertation
Department of Clinical Sciences Division of Oncology and Pathology Skåne University Hospital, Lund, Sweden	Date of issue 13th of May 2016
Author Jacob Elebro	Sponsoring organization

Title and subtitle

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Abstract

Periampullary adenocarcinoma, including pancreatic cancer, has a poor prognosis that has not improved in the last decades. Therefore, in order to find more effective treatment regimens, it is necessary to gain more insight into the biology and clinical behaviour of these tumours.

This thesis entails a thorough histopathological characterization of retrospectively collected tumours from a consecutive cohort of patients with resected periampullary adenocarcinoma, followed by tissue microarray-based immunohistochemical studies of eight candidate protein biomarkers. Tumours were classified as being of pancreatobiliary type (PB-type) or intestinal type (I-type), using histological criteria, and all biomarker analyses were performed in strata according to morphological type.

In Paper I, histopathological studies showed that a standardized and meticulous protocol for assessment of the surgical specimens, as well as blind revisions of slides, had impact on the decision on tumour origin, the number of involved lymph nodes and involved margins.

The biomarker studies in Paper II revealed that expression of the global gene regulator special AT-rich sequence-binding protein 1 (SATB1) was an independent factor of poor prognosis in PB-type tumours. SATB1 expression, however, also indicated a better response to adjuvant chemotherapy, in particular in I-type tumours. A closely related protein, SATB2, was found to be expressed in a few tumours only, making it difficult to draw any firm conclusions on its prognostic value.

In Paper III, biomarker studies on proteins related to gemcitabine metabolism revealed that a high ratio between cytoplasmic and nuclear expression of human protein R (HuR) indicated resistance to chemotherapy in PB-type tumours. In I-type tumours, high expression of human equilibrative nucleoside transporter 1 (hENT1) was a favourable prognostic factor and high expression of deoxycytidine kinase (dCK) indicated sensitivity to chemotherapy.

In Paper IV, biomarker studies on the human epidermal growth factor receptors 1-3 (HER1-3) revealed a potential negative predictive effect of high EGFR (HER1) expression in relation to adjuvant chemotherapy in PB-type tumours. Six percent of I-type tumours had high expression of HER2, and gene amplification was confirmed in assessable cases. In I-type tumours, high expression of HER3 was a favourable prognostic factor, but not independent of other prognostic factors.

Several of the potentially treatment predictive associations described in this thesis are novel, and may be of clinical interest if the results can be repeated in other cohorts and if the mechanistic basis is understood. The results presented here must be however be interpreted with caution, since the cohort is retrospective and many tests have been made.

Key words: Periampullary adenocarcinoma, pancreas, bile duct, ampulla, duodenum, immunohistochemistry, tissue microarray, prognosis

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language: English
ISSN and key title: 1652-8220	ISBN 978-91-7619-274-0
Recipient's notes	Number of pages:83 / Price
	Security classification

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The research presented in this thesis was supported by:

The Swedish Research Council, the Swedish Cancer Society, the Gunnar Nilsson Cancer Foundation, the Mrs Berta Kamprad Foundation, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and University Hospital Research Grants.

Cover photo by Jacob Elebro

Lund University, Faculty of Medicine Doctoral Dissertation Series 2016:48
ISBN 978-91-7619-274-0
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2016

“It is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts.”

Arthur Conan Doyle, Sherlock Holmes

“If facts conflict with a theory, either the theory must be changed or the facts.”

Benedict Spinoza

“If the facts don't fit the theory, change the facts.”

Albert Einstein

Contents

Original papers

The thesis is based on studies reported in the following papers, and are referred to in the text by their respective Roman numerals:

I. Elebro J, Jirström K. Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma. *Diagnostic Pathology* **9**:80 (2014)

II. Elebro J, Heby M, Gaber A, Nodin B, Jonsson L, Fristedt R, Uhlén M, Jirström K, Eberhard J. Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. *Journal of Translational Medicine* **12**:289 (2014)

III. Elebro J, Ben Dror L, Heby M, Nodin B, Jirström K, Eberhard J. Prognostic effect of hENT1, dCK and HuR expression by morphological type in periampullary adenocarcinoma, including pancreatic cancer. *Acta Oncologica* **55**:286-96 (2016)

IV. Elebro J, Heby M, Warfvinge CF, Nodin B, Eberhard J, Jirström K. Expression and prognostic significance of human epidermal growth factor receptors 1, 2 and 3 in periampullary adenocarcinoma. *PLOS ONE* **11**:e0153533 (2016)

Related papers not included in the thesis

- Fristedt R, Elebro J, Gaber A, Heby M, Yudina Y, Nodin B, Uhlén M, Eberhard J, Jirström K. Reduced expression of the polymeric immunoglobulin receptor in pancreatic and periampullary adenocarcinoma signifies tumour progression and poor prognosis. *PLOS ONE* **9**:e112728 (2014)

- Heby M, Elebro J, Nodin B, Jirström K, Eberhard J. Prognostic and predictive significance of podocalyxin-like protein expression in pancreatic and periampullary adenocarcinoma. *BMC Clinical Pathology* **15**:10 (2015)

Abbreviations

APC	Adenomatous polyposis coli
BRAF	V-raf murine sarcoma viral oncogene homolog B
CRT	chemoradiotherapy
dCK	Deoxycytidine kinase
EGFR	Epidermal growth factor receptor
FAP	Familial adenomatous polyposis
FOLFIRINOX	folinic acid, 5-FU, irinotecan and oxaliplatin
FOLFOX	folinic acid, 5-FU and oxaliplatin
GEMCAP	gemcitabine and oral 5-FU prodrug capecitabine
GNAS	G-protein alpha subunit
hENT1	Human equilibrative nucleoside transporter 1
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HR	Hazard Ratio
HuR	Human antigen R
IHC	Immunohistochemistry
ISH	In situ hybridization
IPMN	Intraductal papillary mucinous neoplasm
I-type	Intestinal type
KRAS	Kirsten rat sarcoma viral oncogene homolog
MSI	Microsatellite instability
nab	nanoparticle albumin-bound
NGS	Next-generation sequencing

NRAS	Neuroblastoma RAS viral oncogene homolog
OS	Overall survival
PB-type	Pancreatobiliary type
PFS	Progression-free survival
RFS	Recurrence-free survival
SATB1	special AT-rich sequence-binding protein 1
SATB2	special AT-rich sequence-binding protein 2
SMA	Superior mesenteric artery
SMV	Superior mesenteric vein
SISH	Silver in situ hybridization
TMA	Tissue microarray
wt	Wild type
5-FU	Fluorouracil

Introduction

Periampullary adenocarcinoma

The assessment of tumour origin is central for the traditional classification of adenocarcinomas, and primary adenocarcinomas in the periampullary region can be of pancreatic, distal bile duct, ampullary or duodenal origin. When a patient presents with icterus and an expansive mass in the head of the pancreas, the anatomical origin the tumour is however often not clinically evident, and the majority of periampullary adenocarcinomas are not resected, due to locally advanced and/or metastatic disease. The concept of periampullary describes the anatomical location around the ampulla of Vater, and is useful preoperatively and in cases when resection is not an option.

Epidemiology

Pancreatic cancer is the most common type of periampullary adenocarcinoma, accounting for 3% of all cancer in the USA (1) and the Nordic countries (2) and, due to very high lethality (3), 7% of all cancer-related deaths, making it the fourth most common cause of death in the western world (1, 2). The incidence of pancreatic cancer is slowly increasing in the USA, at approximately 1% each year (1), but the incidence has been fairly unchanged during the last 10 years in the Nordic countries (2). Estimations of future incidence and death from cancer, based on data from the USA, conclude that pancreatic cancer may become the third most common cause of cancer related death in 2020 and the second most common in 2030 (4).

The yearly incidence of pancreatic adenocarcinoma in Sweden is approximately 1000 (2, 5). Other periampullary adenocarcinomas are less common and they are not grouped separately in the national cancer statistics. Each year, there are approximately 400 new cases of cancer in the bile ducts (including the distal bile duct and ampulla of Vater, but also the gall bladder) (2).

European data show an annual incidence of 16.6 per million of extrahepatic bile duct cancer (excluding gallbladder and ampulla of Vater) and 5.7 per million of

small bowel cancer. For Sweden, with a population of approximately 10 million, these numbers correspond to 166 and 57 new cases of extrahepatic bile duct cancer and small bowel cancer per year (6).

Aetiology

Life style and environmental factors

Environmental factors in periampullary cancer are mostly studied in pancreatic cancer. The relative risk of pancreatic cancer among smokers is 1.74, as compared with non-smokers (7) and it has been estimated that 23% of pancreatic cancer is attributable to tobacco smoking (8). Studies on a possible increase in risk from use of Swedish moist snuff (snus) are few but have indicated that ever-users of snus, as compared with never-users of any tobacco, have a relative risk of 2.0 for pancreatic cancer (9) and 1.6 for any smoke-related cancer (10), and that ever-users of snus, as compared with never-users of snus, have no increase in risk (9, 10). Another study found a relative risk of 1.7 among ever-users, as compared with never-users of snus, but, on the other hand, very few cases of pancreatic cancer among never-smokers (11). Taken together, these results suggest that snus increases the risk for pancreatic cancer, but also illustrate that smoking is a major cofounder.

Heavy alcohol consumption increases the risk of pancreatic cancer by 1.6, as compared with no alcohol consumption (8).

Chronic pancreatitis increases the risk of pancreatic cancer by 11, but this association is not independent for pancreatitis with duration of more than 2 years, due to a strong association to smoking and heavy drinking (8).

Type II diabetes is both a cause of and a consequence of pancreatic cancer and the exact risk increase in persons with type II diabetes is therefore difficult to assess. The overall increase in risk of pancreatic cancer in patients with type II diabetes is 1.8, but the increased risk is mainly observed during the first years after the onset of diabetes, thus likely reflecting cases where diabetes is a consequence and not a cause of cancer (12).

A register-based study on type I diabetes and risk of pancreatic cancer found a similar increase in risk of pancreatic cancer as in type II diabetes (13), whereas a study including only patients hospitalized with type I diabetes before the age of 30 found no increase in risk (14). This discrepancy suggests that misclassification of type II as type I diabetes can produce a false increase in risk of pancreatic cancer.

Overweight and obesity increase the risk of several types of cancer, including pancreatic cancer (15). Dietary patterns (high fruit vs high fat) have, on the other hand, not shown any clear associations with risk of pancreatic cancer (16).

Thorotrast, a radioactive X-ray contrast medium used from 1930 to 1950 increased the risk of intra- and extrahepatic bile duct cancer, as well as other malignancies (17).

Gallstones increase the risk of cancer of the gallbladder, but bile duct stones have not been linked to development of cancer in the extrahepatic bile ducts or pancreas, although some studies indicate an increased risk of pancreatic cancer (18).

Liver fluke, transmitted by eating undercooked infected fish, is common in East Asia, and increases the risk of bile duct cancer, especially intrahepatic (19).

Germline mutations

Five to 10% of pancreatic cancer is estimated to be hereditary (20), and having one or two first degree relatives with pancreatic cancer increases the risk by 4.6 and 6.4 respectively (21). In approximately 80% of familial pancreatic cancer, the genetic basis has not yet been found, but several inherited autosomal dominant mutations are known to increase the risk of pancreatic adenocarcinoma (22, 23).

BRCA2 and BRCA1

BRCA2-mutations are the most common germline mutations in familiar pancreatic cancer, and they are seen in 6% of families with a moderately increased incidence of pancreatic cancer, and in 17% of families with a high risk of pancreatic cancer (24-26). Germline *BRCA2* mutation increases the risk of pancreatic cancer by 3-6 (27-29) and bile duct/gallbladder cancer by 5 (27). Approximately 2-5% of *BRCA2* mutation carriers are diagnosed with pancreatic cancer (29).

Mutations in *BRCA1* have been found to increase the risk of pancreatic cancer by 2-3 (29, 30), and 3-4% of carriers are diagnosed with pancreatic cancer (29).

The increased risk of pancreatic cancer in carriers of *BRCA1/2* mutations is similar in men and women (31).

In a consecutive clinic-based cohort of 306 unselected cases of pancreatic cancer there were 3.6% germline mutations in *BRCA2* and 1.0% in *BRCA1* (32), and the majority of patients with these mutations did not meet the criteria for genetic testing, although a family history of pancreatic cancer was overrepresented, which suggests that genetic testing must be performed in more patients with pancreatic cancer in order to find the hereditary forms. The incidence of *BRCA2* mutations in

unselected cases of pancreatic cancer might however be somewhat lower, as the COSMIC database reports 1% with mutations of *BRCA2*, and 1% of *BRCA1* (33). It has been observed that not all families with *BRCA2*-mutations and familial pancreatic cancer have an increase in breast or ovarian cancer (24, 26). Biallelic *BRCA2*-inactivation is a relatively late event in pancreatic carcinogenesis in patients with germline *BRCA2*-mutations, which might explain why not more of the mutation carriers develop pancreatic cancer (34). The increased risk of pancreatic cancer in *BRCA* mutation carriers is however greater before than after the age of 65, indicating an earlier onset in carriers than in the general population (27).

PALB

The second most commonly mutated gene in hereditary pancreatic cancer is in the tumour suppressor *PALB*, seen in 3-4% of cases, which codes for a protein that links BRCA1 and BRCA2 together (35, 36).

ATM

ATM collaborates with BRCA1 in DNA double-strand break repair. Germline mutations in the *ATM* gene have been found in 2-5% of families with hereditary pancreatic cancer (23, 37).

CDKN2A

The familial atypical mole and multiple melanoma (FAMMM) syndrome is caused by mutations in the *CDKN2A* gene which codes for both p16 and p14arf. For affected individuals, the estimated lifetime risk of malignant melanoma is 60-90% and 20% for pancreatic cancer (22). Correspondingly, germline mutations in *CDKN2A* increase the risk of pancreatic cancer by factor 22 (38).

Hereditary pancreatitis

Cationic trypsinogen protein is highly expressed in acini, and germline mutations in its gene *PRSS1* increase the risk for acute pancreatitis, due to premature protein activation, and subsequent chronic pancreatitis. The risk of pancreatic cancer in carriers of germline *PRSS1* mutations is 35 times higher than in the general population (22). Germline mutations in other genes have been described in hereditary pancreatitis, including genes coding for Chymotrypsin C (*CTRC*), serine protease inhibitors of the Kazal type (*SPINK*) and cystic fibrosis transmembrane receptor (*CFTR*) (22).

Peutz-Jeghers syndrome

Peutz-Jeghers syndrome, with germline mutations in the *STK11* gene, is characterized by gastrointestinal hamartomas and mucocutaneous pigmentations,

but also increases the risk of pancreatic and periampullary malignancies. It has been estimated that the lifetime risk of periampullary malignancy (including adenocarcinoma, neuroendocrine tumours and acinar cell carcinoma) is 32%, with an increase in risk by factor 96 (39).

FAP

Carriers of germline inactivating mutations in the *APC* gene have a virtually 100% lifetime risk of colorectal cancer that can be circumvented by prophylactic proctocolectomy. The patients still have a very high risk of duodenal adenomas and a cumulative 10% risk of periampullary adenocarcinoma at the age of 60 (40). Cases of IPMN, neuroendocrine tumours, pancreatoblastoma, acinar cell carcinoma, common bile duct polyposis and pancreatic desmoid tumours have been reported in association with FAP, but regular pancreatic adenocarcinoma does not seem to be overrepresented (41).

Lynch syndrome

Lynch syndrome, caused by germline mutations in mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) increases the risk of colorectal and endometrial cancer, but also of several other cancers. Small bowel adenocarcinoma is uncommon in the general population, but after colorectal cancer due to Lynch syndrome the risk is increased to 4% during the following 20 years (42), and Lynch patients have a relative risk of 73-100 (42, 43). Lynch patients also have an increased risk of hepatobiliary tract cancer, with a relative risk of 6 (42). The life time risk of pancreatic cancer is 9 times higher than in the general population (44). In series of small bowel adenocarcinoma, microsatellite instability (MSI) has been found in 18-35% (45-47) and evidence of Lynch syndrome in two thirds of MSI cases (47).

Prognosis

There has been no substantial improvement in survival of pancreatic cancer in the last 40 years (5). One year overall survival (OS), for all stages combined, is 19% (48). Patients with locally advanced or metastatic pancreatic cancer have a median OS of approximately 6 months (49-53). Resectable patients have a median OS of approximately 24 months after resection (54).

Patients with ampullary adenocarcinoma have a median OS of 36-44 months after resection (55-57).

For extrahepatic bile duct cancer of all stages combined, the 1- and 5-year OS in Europe is 36% and 11%, respectively (6). Median OS after resection for distal bile duct adenocarcioma is similar or slightly longer than for resected pancreatic

adenocarcinoma (55), but long term survival is better than for pancreatic cancer, with reports on 30% surviving 5 years after a pancreatoduodenectomy (58).

For small bowel cancer, the 1- and 5-year OS in Europe is 49% and 23%, respectively (6). After resection for duodenal adenocarcinoma, 1- and 5-year OS is around 90% and 60%, respectively (59).

Early detection

Excess death within 1 and 3 months after diagnosis of pancreatic cancer or small bowel cancer decreased from the 1960´s until the 1990´s, but then reached a plateau (5). Better survival during the first period after diagnosis, but not a corresponding increase in long term survival, can be attributed to improved surgical technique and slightly earlier detection. To improve survival from pancreatic cancer by early detection, one would need to find the tumours at an earlier tumour stage than today, since small tumours without lymph node metastases have a substantially longer survival and approximately one third are cured by surgery (60). Series of case reports of very early pancreatic cancer, measuring less than 10 mm, indicate a five year OS of 57% (61) and a better survival in cases with fewer symptoms and signs of the tumour. A five-year OS of 57% is extremely high in the context of pancreatic cancer, and highlights the need for early detection. Since the incidence of pancreatic cancer is low, current methods of detection are not suitable for screening of the general population, but may be feasible in high-risk groups (62).

Although *KRAS* mutations are common and early events in pancreatic cancer, they are also found in benign conditions and thus not a reliable marker for early detection. Analysis of pancreatic juice in 54 patients with chronic pancreatitis (median age 55 years) showed *KRAS* mutations in 37%, but none of the patients developed pancreatic cancer during a 6.5 year follow up time (63). Also, an autopsy study on patients without chronic pancreatitis or pancreatic cancer showed hyperplastic epithelium of the main duct in 86% and *KRAS* mutations in 32% of cases with hyperplastic epithelium, however often different mutations in codon 12 than in pancreatic cancer (64).

Type II diabetes may be a consequence of pancreatic cancer (12), and older patients with new-onset type II diabetes have an 8 times higher risk of pancreatic cancer than the general population(62), but new tools or markers are needed to differentiate type II diabetes due to pancreatic cancer from other type II diabetes. Exosomes containing adrenomedullin, shed from pancreatic cancer into the circulation, seem to cause type II diabetes in pancreatic cancer by a paracrine

mode of action, and might become useful for separating paraneoplastic from regular type II diabetes (65).

In the context of pancreatic cancer, CA19-9 is the most studied biomarker in blood, but its use is limited to symptomatic patients, and does not aid in early detection and screening of non-symptomatic individuals (66).

Mathematical simulations of the effect of prevention, early detection and treatment of colorectal cancer attribute most of the decline in death from colorectal cancer to early detection, and less to prevention and treatment advances (67). Computational models on the development and spread of pancreatic cancer estimate that the time from the first genetic event to invasion, metastasis and death is approximately 12 + 7 + 3 years (68), which suggests that earlier detection could change the course of the disease.

Radiology

Ultrasound has a high sensitivity (87%) and specificity (99%) for detecting malignant lesions in the head of the pancreas, but is user dependent (69). Computed tomography (CT) optimized for pancreas, with intravenous contrast, generally gives more information on local extension into the superior mesenteric vein (SMV), superior mesenteric artery (SMA) or celiac axis, and metastases that disqualify for resection (70). Magnetic resonance cholangiopancreatography can give further preoperative information (71).

Treatment

Surgery

Surgical strategies to resect parts of the duodenum and pancreatic head were first explored in the end of the 19th and beginning of the 20th century, and one-stage pancreaticoduodenectomy was later developed by Whipple, who became the eponym for the procedure (72).

Mortality and morbidity related to the procedure was very high during the 1950´s to 1970´s, with reports of 25-40% mortality during the hospital stay (73-75), but decreased substantially in the 1980´s with perioperative mortality rates around 1-4% (76, 77) and several more recent reports from high-volume centres describe 1-2% 30-day mortality (55, 78-80). Postoperative complications and mortality is also

significantly lower in high volume centres, as compared with low volume centres (81), and both hospital volume and surgeon volume seem equally important (82).

The pancreatoduodenectomy procedure is an extensive operation in which the duodenum, the head of pancreas and distal parts of the extrahepatic bile ducts are removed. In selected cases, resections of the superior mesenteric vein (SMV) can be done, to achieve macroscopically radical resections. Adherent to the surfaces of the specimen are lymph nodes and peripancreatic fat, which are important for staging of the tumour.

Surgical resections of periampullary tumours are possible, with a curative intent, in 15-20% of patients (49, 83).

Chemotherapy

Neoadjuvant chemotherapy

Approximately one third of all pancreatic cancer is locally advanced at presentation, and although the borderline resectable subgroup is often ill-defined or poorly represented in studies on neoadjuvant therapy there is a consensus that some tumours can be converted to resectability (50). Neoadjuvant treatment has often been based on 5-FU or gemcitabine, with or without radiotherapy. Best results in the metastatic setting has been achieved, from a triple combination including 5-FU, irinotecan and oxaliplatin (FOLFIRINOX) or a double combination including gemcitabine and nab-paclitaxel (nanoparticle albumin-bound paclitaxel), and these two regimens are assumed to be more effective also in the borderline resectable neoadjuvant setting (84).

Patients who are resected after neoadjuvant treatment for borderline resectable pancreatic adenocarcinoma have a doubled OS compared with those who remain non-resectable, and the survival approaches that of the upfront resectable patients (50).

Adjuvant chemotherapy

Randomized trials in the 1990´s showed that adjuvant chemotherapy for pancreatic cancer prolongs life, as compared to observation (85, 86). After better results from gemcitabine than from 5-FU in the palliative setting (53), gemcitabine is often the preferred adjuvant agent in Europe, although 5-FU and gemcitabine have been comparable in studies on adjuvant therapy in pancreatic cancer (54, 87).

In Japan, the oral 5-FU prodrug S-1 is considered more effective than gemcitabine in the adjuvant setting (88), but is less studied in the west.

The effect of chemotherapy in ampullary and distal bile duct carcinoma is not as well studied as in pancreatic cancer. The large ESPAC-3 study on resected periampullary adenocarcinoma, however, found a survival benefit from adjuvant gemcitabine, but only after adjusting for adverse factors in multivariable analysis (89). This result has still provided some evidence for the use of adjuvant gemcitabine in ampullary and distal bile duct cancer, but it is still uncertain whether patients with resected duodenal adenocarcinoma benefit from adjuvant chemotherapy (90).

In the ESPAC-3 trial there was no evidence that neither pancreatobiliary type (PB-type) nor intestinal type (I-type) of ampullary adenocarcinoma respond better to either gemcitabine based or 5-FU based adjuvant chemotherapy (89), but differences in prognosis and molecular profiles still suggest that PB- and I-type ampullary adenocarcinoma should be regarded as discrete entities (91, 92).

In the ABC-02 study on advanced biliary tract adenocarcinoma, including ampullary adenocarcinoma, there was a significantly longer survival in the group that received gemcitabine and cisplatin as compared with only gemcitabine, but there was no survival difference in the subgroup of ampullary adenocarcinoma (93). After this study gemcitabine combined with a platinum based compound is an accepted adjuvant treatment in distal bile duct adenocarcinoma.

The ESPAC-4 study, which is ongoing, compares adjuvant gemcitabine to a combination of gemcitabine and oral 5-FU prodrug capecitabine (GEMCAP) in periampullary adenocarcinoma, including pancreatic cancer (94).

Palliative chemotherapy

The first report on prolonged survival from chemotherapy in advanced pancreatic cancer came from a randomized trial in 1980 (95). Clinical trials in the mid 90´s, on patients with non-resectable pancreatic cancer, showed that single therapy gemcitabine relieved symptoms better and also prolonged life as compared to single therapy 5-FU (53). Thereafter, gemcitabine has become standard chemotherapy for pancreatic cancer in Europe, both in the adjuvant and in the palliative setting. In Japan, the oral 5-FU prodrug S-1 is considered equally effective as gemcitabine in the palliative setting (88), and a small phase II clinical study in Europe, on S-1 in metastatic pancreatic cancer showed results comparable to those of gemcitabine (96).

Most combinations of gemcitabine and other chemotherapy agents have not improved survival or quality of life compared with gemcitabine alone (97), and gemcitabine has therefore for a long period been standard treatment. During the last years some alternative combination chemotherapies have shown better progression-free survival (PFS) and/or OS, such as GEMCAP (gemcitabine and 5-FU prodrug capecitabine) (98), and combinations with platinum based compounds

(99) FOLFOX (folinic acid, 5-FU and oxaliplatin) and FOLFIRINOX (folinic acid, 5-FU, irinotecan, and oxaliplatin), and nab-paclitaxel combined with gemcitabine, which all have shown superiority to gemcitabine (51, 52, 100). Relative toxicity, especially from FOLFOX and FOLFIRINOX, has however limited these combinations to patients with a good performance status (51).

Targeted therapy

Receptor tyrosine kinase inhibitor erlotinib, combined with gemcitabine, is the only targeted therapy that has increased PFS or OS in pancreatic cancer in a phase III trial, but the benefit from adding erlotinib was modest (101). Other phase III trials have all combined gemcitabine with one targeted agent, e.g. monoclonal antibodies against EGFR, type 1 insulin-like growth factor receptor (IGF-1R) or vascular endothelial growth factor A (VEGF-A), without improved survival compared with gemcitabine alone. Several phase I and II trials are ongoing, targeting various pathways. Only 8% of the most recently registered trials stratify patients using a biomarker and few target more than one point in a cascade (102).

Radiotherapy

Neoadjuvant radiotherapy

Radiotherapy in combination with chemotherapy is used for borderline resectable pancreatic cancer, and up to a third become resectable (50).

Adjuvant radiotherapy

There are conflicting results, and views, on the value of adding radiotherapy to adjuvant chemotherapy in the treatment of resected pancreatic cancer, and it seems as if chemoradiotherapy (CRT) is favoured in North America, while chemotherapy is favoured in Europe. A large register based study found prolonged survival after adjuvant CRT, as compared with chemotherapy (103), while a large meta-analysis found no such benefit, but instead increased toxicity compared with chemotherapy (104).

Palliative radiotherapy

Palliative radiotherapy for locally advanced pancreatic cancer is controversial as it is believed to alleviate local symptoms, but may cause side effects without treating microscopic metastases, which are common (105). In a recent study, induction therapy with gemcitabine was given to patients with locally advanced tumours without signs of metastases, and after the induction patients were randomised to continuing with gemcitabine or receiving CRT. The study showed no benefit from

CRT, however, it´s still not clear if there are subgroups in this setting that may benefit from CRT (106). After this trial, CRT is used with caution in selected patients with locally advanced pancreatic cancer.

Most patients with metastatic pancreatic cancer have many metastases, and oligometastatic spread is uncommon. Radiotherapy can however relieve symptoms in oligometastatic spread to bone, and possibly also prolong OS in oligometastatic spread to the liver (107).

Personalized therapy

In standard clinical practice, there is yet no biomarker that is used to stratify patients with periampullary adenocarcinoma for treatment with different chemotherapeutic agents or targeted therapy. There are however several theoretically druggable aberrations in pancreatic cancer including KRAS, CDKN2A, ARID1A, BRCA1/2, PALB2, PIK3CA and BRAF (108).

BRCA1/2 mutations, PALB2 mutations and loss of ATM

The genome of pancreatic cancer can be very unstable, which leads to heterogeneity within a single tumour and among its metastases (109). Such genetic heterogeneity makes it difficult to find effective targets (102), but genomic instability, as a sign of deficient repair, can also be a target (110). Retrospective observations indicate that patients with germline *BRCA* mutations respond unusually well to either platinum based chemotherapy, that cross links DNA, or Poly(ADP-ribose) polymerase (PARP)-inhibitors, due to inefficient DNA-repair (111-113). PARP 1&2 proteins detect and bind to single strand DNA breaks, with subsequent DNA-repair, and PARP inhibitors thus cause single strand breaks to evolve into double strand breaks, which is cytotoxic in cells without functional BRCA1 or -2. Tumours with wild type BRCA and inefficient DNA-repair through other mechanisms, such as loss of ATM, also show increased sensitivity to PARP-inhibition (114). Results from several phase II studies with PARP-inhibitors in mainly breast and ovarian cancer with *BRCA* mutations are promising, and phase III trials are ongoing (115), and similarly good responses have been seen in germline *BRCA* mutations and pancreatic cancer (116). Studies have found *BRCA* mutations in 4% of pancreatic cancer, but whole genome sequencing has shown that 14% of pancreatic cancer has a highly unstable genome, thus increasing the number of patients that could benefit from platinum based chemotherapy or PARP-inhibition (110). PALB2 binds to BRCA2 and thus also has a role in DNA repair. It is mutated in 3% of familiar pancreatic cancer (35), but only in 0.4% of pancreatic cancer in general (33).

HER2 mutations and amplifications

NGS has revealed several activating and druggable *HER2* mutations, in addition to amplifications, that seem to be particularly common in duodenal and extrahepatic bile duct cancer, with a frequency of approximately 25% and 20%, respectively (117), and trials on *HER2*-mutated cancer are ongoing.

KRAS mutations

Inhibition of MEK, a protein kinase downstream of KRAS, has shown response in a few cases of advanced pancreatic cancer in early phase trials, but the overall effect has not been better than from gemcitabine (108).

ARID1A

ARID1A mutations frequently co-occur with increased activity in the PI3K/AKT-pathway, and indicate an increased sensitivity for AKT-inhibitors and PI3K-inhibitors (118).

PIK3CA

PIK3CA mutations cause constitutive activation of the PI3 kinase and downstream activation of the AKT pathway. As a consequence, COX-2 is upregulated, and *PIK3CA* mutations could thus theoretically identify potential responders to COX inhibition. In a retrospective study, regular intake of aspirin, but not the NSAID rofecoxib, was associated with a prolonged recurrence-free survival (RFS) in resected colorectal cancer with *PIK3CA* mutation, compared with wild type (119).

Activation of the AKT-pathway, by *PIK3CA* mutations, could theoretically also be targeted by inhibitors of AKT.

BRAF

Inhibition of mutated *BRAF* (V600E) is a successful biomarker guided treatment in metastatic malignant melanoma, but not in V600E mutated colorectal cancer, due to upregulation of EGFR after *BRAF*-inhibition, through activation of MEK. Consequently, simultaneous inhibition of BRAF and EGFR, or MEK and EGFR, has inhibited cell growth and induced apoptosis in V600E mutated colorectal cancer (120). Ongoing trials on colorectal cancer show promising results from combinations of MEK-inhibitor, BRAF-inhibitor and anti-EGFR antibody (121).

P53 and Wee1 inhibition

Tumours with inactivated P53 have deficiencies in the G1-S checkpoint, and therefore depend more on the G2-M checkpoint to halt cell division. Wee1 counteracts G2 transition by inhibitory phosphorylation of cyclin-dependent kinase 1, and Wee1-inhibition can thus cause premature mitotic entry and increased vulnerability to DNA-damaging agents in P53 deficient tumours (122).

P53 and MDM2 inhibition

Tumours with wild type p53 often have increased levels of MDM2 that inhibit the tumour supressing features of p53 (123). Several different MDM2 inhibitors are in early phase clinical trials (124), and seem most effective in tumours with amplification of the *MDM2* gene. Amplification of *MDM2* has been found in 7-10% of cases in a wide range of malignancies, most frequently in sarcomas (125, 126). *MDM2* amplification has been shown in 9% of colorectal cancer, with an association to metastatic disease (127) and in 42% in a small cohort of gastric cancer (128), but not in pancreatic cancer (125).

Mutations, deletions and amplifications

The most commonly reported mutated genes in pancreatic cancer are the oncogene *KRAS*, and the tumour suppressors *P53*, *CDKN2A*, and *DPC4* (108). During the development of preinvasive pancreatic neoplasia, *KRAS* mutations and *HER2* overexpression are the earliest alterations, followed by p16 inactivation and later loss of function of p53, DPC4 and BRCA2 (129). In addition to inactivating mutations, the tumour suppressor genes are commonly inactivated by deletions and promoter methylations.

Mutations are more common in pancreatic cancer of smokers as compared with non-smokers, but not in the major driver genes (130).

KRAS

Activating *KRAS* mutations are considered very common in pancreatic cancer, and based on studies with mutations in 71-93% (131-134), it is often said that 90% of pancreatic cancer cases harbour *KRAS* mutations (135, 136). However, the largest collection of mutation data, the COSMIC database, reports a lower incidence; 70% out of 5832 cases (33). This database also reports *KRAS* mutations in 29% of ampullary adenocarcinoma, while a cohort of 140 ampullary adenocarcinomas had activating *KRAS* mutations in 40% (137). The COSMIC database further describes *KRAS* mutations in 24% of bile duct and 32% of duodenal adenocarcinoma (33). In a large cohort with small bowel adenocarcinoma, *KRAS* mutations were seen in 43% of cases (138).

KRAS protein bound to guanosine triphosphate (GTP) gives signals that regulate cell activities such as proliferation, differentiation, apoptosis, and cell migration (139). Mutated, oncogenic KRAS has a key role in the initiation of pancreatic cancer (129). Activating *KRAS*-mutations are however not sufficient for transformation, as exemplified by mutated KRAS found in healthy individuals and

KRAS-mutated mice developing tumours from only a fraction of the affected cells. The KRAS activity thus has to reach a certain level before it can lead to neoplasia, which is supported by experiments demonstrating that upstream signalling from e.g. EGFR can increase the rate at which a tumour is formed, even when KRAS is constitutionally active (140). There are also examples of small bowel adenocarcinoma with concurrent activating mutations in *KRAS* and *HER2* (138).

KRAS mutations in codon 12 have been demonstrated to be an adverse factor for OS in advanced pancreatic cancer, as compared with wild type *KRAS*, but not predictive in regard to treatment with erlotinib (141), and specific *KRAS* mutations in codon 12 have been associated with a shorter OS in resected pancreatic adenocarcinoma (142).

P53

Inactivating mutations in the tumour suppressor *P53* are found in 44% of pancreatic, 34% of bile duct and 27% of duodenal adenocarcinoma in the COSMIC database, but are less studied in ampullary adenocarcinoma (33). In small bowel adenocarcinoma, *P53* mutations are seen in 41% of cases (138). Inactivating *P53* mutations lead to loss of its anti-proliferative properties, but also to gain of metastatic potential, by enabling transcription of platelet-derived growth factor receptor b (PDGFRb) (143). In addition to frequent mutations, wild type *P53* is inactivated in many cancers by MDM2 through either MDM2 gene amplification or posttranscriptional stabilization (144). *P53* mutations are associated with poor prognosis in several types of cancer (145), as well as resistance to chemotherapy (146).

CDKN2A

CDKN2A is the most frequently altered tumour suppressor gene in pancreatic adenocarcinoma, with loss of protein function in 90% of cases, due to promoter methylation, biallelic loss or mutation and loss of the other allele (147-149).

Mutations in the *CDKN2A* gene are seen in 13% of pancreatic, 17% of biliary tract and 23% of ampullary adenocarcinoma in the COSMIC database (33), affecting its two most known gene products, the tumour suppressors p16 and p14arf, that have a regulatory role in the cell cycle by stabilizing the tumour suppressor Rb (retinoblastoma) protein and activating P53 by inhibiting MDM2 (150).

Loss of P16 has been shown to be prognostic in colorectal cancer, but not independent of general hyper-methylation of promoters (151).

DPC4

Loss of DPC4 expression is seen in 50% of cases of pancreatic cancer, with inactivating mutations in 20% (108), and deletions in another 30% (152). DPC4 loss is considered less common in other tumour types (152), but immunohistochemical (IHC) evidence of loss is seen in approximately 50% of other periampullary adenocarcinoma (153) and distal bile duct adenocarcinoma (154), but without significant associations with prognosis. In a cohort of ampullary adenocarcinoma, there was IHC loss of DPC4 in 36% of I-type tumours and in 40% of PB-type tumours, indicating no obvious difference between the morphological types regarding DPC4 (137). *DPC4* mutations have also been described in 10% of small bowel adenocarcinoma (138).

Signalling in the transforming growth factor beta (TGF beta) pathways has both oncogenic and tumour suppressing features, with the latter often being inactivated in cancer (155), and loss of the tumour suppressor DPC4 is one of the mechanisms behind the increased oncogenic properties of TGF beta (155, 156). Loss of DPC4 increases the metastatic potential (157), and is an independent factor for shorter RFS and OS in resected pancreatic cancer (158). IHC assessment of DPC4 status mirrors inactivation at the genetic level, and, negative IHC staining can be regarded as a marker of metastatic potential (153). Tumours with loss of DPC4 expression often have missense mutations in *P53*, suggesting that during carcinogenesis, some remaining P53 activity can select for DPC4 loss (159).

Less frequent genetic events

CCND1, an oncogene coding for cyclin D1, is overexpressed in many types of cancer where it facilitates cell cycle progression, decreased dependence on extracellular anchoring signals and overriding of the effect of inhibitory proteins such as P16 (145). Overexpression of cyclin D1 has been described in 70-80% of pancreatic cancer, corresponding to a shorter survival (146). *CCND1* amplification has been reported in 7% of ampullary tumours of both morphologies (147).

HER3 mutations have been described in 11% of ampullary adenocarcinoma, equally distributed between PB- and I-type tumours (91), but only in 1% of extrahepatic bile duct cancer (160), and even more seldom in pancreatic cancer (33).

Activating *HER2* mutations have been found in 16% of duodenal adenocarcinoma, but only in a few percent of more distal small bowel adenocarcinoma (138). *HER2* mutations have been seen in 4% of extrahepatic bile duct cancer (160), and in 1% of pancreatic cancer (33).

The tumour suppressor *ELF3* has been found to be mutated in 15% of ampullary adenocarcinoma, and to be mutually exclusive with *CDKN2A* mutations (91). *ELF3* mutations have also been found in 5% of biliary tract adenocarcinoma (160), but seldom in pancreatic cancer (33).

ARID1A is a tumour suppressor that collaborates with p53 to regulate transcription of *CDKN1A* (P21) and *SMAD3* (161). It has been found to be mutated in 4%, and underexpressed in 16% of pancreatic cancer, and mutated in 20% of biliary tract cancer (33).

Mutations in *BRCA2* have been found in 3-4% of unselected cases of pancreatic cancer (29), and in 4% of extrahepatic bile duct cancer (160), but are less examined in adenocarcinomas of the ampulla and duodenum (33).

BRCA1 mutations are described in 1% of pancreatic (33), and 1% of extrahepatic bile duct adenocarcinoma (160).

ATM collaborates with BRCA1 in DNA double-strand break repair, and somatic mutation or loss has been reported in 8% of sporadic pancreatic cancer (134).

PIK3CA mutations are seen in 10% of small bowel adenocarcinoma (138), in 4% of extrahepatic bile duct cancer (160) and in 2% of pancreatic cancer (33).

BRAF mutations are described in 7% of small bowel adenocarcinoma (138), but are uncommon in extrahepatic bile duct cancer (160) and in pancreatic cancer (33).

TGFBR2 mutations are common in colorectal cancer displaying MSI, and have been described in 5.5% of pancreatic adenocarcinoma (142), and in 4% of extrahepatic bile duct cancer (160) but are less examined in duodenal cancer.

EGFR mutations have been reported in 4% of pancreatic cancer (142), but only in 0.4% of pancreatic cancer in the COSMIC database (33).

GNAS mediates the effect of several hormones, and is involved in different hormone-dependent tumours (162). *GNAS* mutations are common in intraductal papillary mucinous neoplasia (IPMN) of the pancreas and in adenocarcinomas associated with IPMN (163), and have also been recorded in 4% of a consecutive series of pancreatic adenocarcinoma (142), and in 4% of small bowel adenocarcinoma (138).

NRAS mutations are described in 1% of small bowel adenocarcinoma (138).

APC is a tumour suppressor that regulates beta catenin concentrations and interacts with E-cadherin. It is commonly inactivated in colorectal cancer. Mutations in *APC* have been seen in 1% of pancreatic adenocarcinoma (142) and in 13% of small bowel adenocarcinoma (138).

Histopathology of periampullary adenocarcinoma

Tumour origin in periampullary adenocarcinoma

The basis for current classification of adenocarcinomas is tumour origin. This assessment is mainly anatomical, since histologic tumour centre is the most reliable sign of tumour origin. Periampullary adenocarcinomas often invade and sometimes fully overgrow adjacent structures, and sometimes also imitate the epithelium of the invaded structure (164), which explains why e.g. cancer growing in the duodenal mucosa is not synonymous with duodenal cancer. Pancreatic cancer is often multifocal in the head of the pancreas, and is often associated with chronic pancreatitis, while the other periampullary adenocarcinomas are often rather well demarcated microscopically, with an obvious tumour centre in or around that particular anatomical structure.

Evidence for a beneficial effect of adjuvant chemotherapy was present for pancreatic adenocarcinoma earlier than for other types of periampullary adenocarcinoma. There was thus a period when tumour origin was the basis for decision if adjuvant chemotherapy would be administered or not. In recent years, resected periampullary adenocarcinomas of different origins can enter study protocols for adjuvant treatment, but tumour origin is still crucial for which study a patient can enter.

In patients with non-resectable tumours, a morphological diagnosis by fine needle aspiration, core needle biopsy or surgical biopsy is necessary before palliative chemotherapy can be given. The main objective in this situation is to establish a cancer diagnosis. In addition to a cancer diagnosis, these often sparse cytological or histological specimens can sometimes help differentiate between PB- and I-type of periampullary adenocarcinoma, but are insufficient for determining tumour origin.

When a periampullary tumour presents without signs of distant spread or overgrowth on nearby structures, thus being resectable, an anatomical and histological assessment of tumour origin can be made on the surgical specimen. Both statistics and reason tell us that pancreatic origin dominates among the non-resectable tumours. In resected periampullary adenocarcinoma, however, assessment of tumour origin is essential for proper stratification of risk, participation in clinical studies and for basic studies on tumour biology and genetic profiles. The assessment of tumour origin can however be difficult (165, 166), and a large variation in proportions of tumour origin between studies on resected periampullary adenocarcinoma indicate that different pathologists evaluate the histopathology differently (167, 168). Blind revisions of slides also

often result in reclassification of tumour origin, with a substantial increase of distal bile duct origin (167). A more extensive pathology protocol also appears to identify a larger proportion of tumours of non-pancreatic origin compared with non-standardized protocol (167-169). The proportion of operated cases with lymph node metastasis (N1) in a series can possibly be used as a surrogate measure for the thoroughness of the pathology examinations. A high percentage of N1 in a cohort also seems to be associated with a higher fraction of non-pancreatic origin (169). Also the fraction of cases with involved margins might serve as a marker for thoroughness, but comparisons between series are not always possible due to different definitions of involved margins. The observation that tumour origin to some degree depends on the pathology assessment, has implications on prognosis, since classification of non-pancreatic adenocarcinomas (especially I-type adenocarcinomas) as pancreatic can "produce" long term survivors. In a very thorough study on 4922 patients that, according to the Finnish Cancer Registry, were diagnosed with pancreatic cancer during a 7-year period, 89 patients (1.8%) survived 5 years after diagnosis. On thorough evaluation of pathology reports and slides, pancreatic cancer could however only be confirmed in 10 cases (0.2%), which suggests that the 5-year survival rate is 0.2% rather than 1.8% (3). These figures are in sharp contrast to data from cancer registers in the USA, with 5-year OS in pancreatic cancer reaching 7% (1). Also cancer statistics from Korea show that between the periods 1993-1995 and 2003-2007, there was an increase in the fraction of patients surviving 5 years in all measured cancer types, except pancreatic cancer, where 5-year survival decreased from 9-10% to 7.6% (170). The cause of this apparent worsening of the prognosis in Korea is not known, but one explanation may be alterations of diagnostic criteria.

Pancreatic adenocarcinoma

Pancreatic adenocarcinoma is often referred to as "ductal cancer" or "ductal adenocarcinoma". From a histological viewpoint it is however not surprising that an adenocarcinoma contains duct-like structures, and there is no particular non-ductal pancreatic adenocarcinoma that one conceptually needs to separate from the "ductal". As a comparison, in breast cancer, the concept of ductal and lobular adenocarcinoma has a histological and molecular foundation, and clinical implications. In this thesis, adenocarcinoma emanating from the pancreas is called pancreatic adenocarcinoma, thus excluding metastases, non-adenocarcinomas and non-invasive pancreatic neoplasms.

Seventy-five percent of pancreatic adenocarcinoma occurs in the head of the pancreas, and the remaining 25% are equally distributed between the body and tail (49). At diagnosis, 50-60% have stage IV disease, with evidence of metastases beyond local lymph nodes; 30-40% has stage III disease, with locally advanced

growth without evidence of metastases and the remaining 10-20% are resectable (49, 50).

In resected pancreatoduodenectomy specimens, pancreatic adenocarcinoma is seldom well demarcated with surrounding microscopically normal pancreatic parenchyma. Instead, these tumours are often intermingled with chronic pancreatitis and often have a multifocal appearance. The tumours often invade the distal bile duct, and sometimes also the papilla of Vater and/or duodenum.

Distal bile adenocarcinoma

A distal bile duct adenocarcinoma operated with pancreatoduodenectomy has its origin in the common bile duct (ductus choledochus) or in the distal part of the common hepatic duct (ductus hepaticus communis). Sometimes these adenocarcinomas are small and localized, most likely due to early detection after disturbance in the flow of bile, but often invade surrounding pancreatic parenchyma. They often grow both circumferentially around and longitudinally along the bile duct.

Ampullary adenocarcinoma

Tumours in the papilla of Vater, which protrudes into the duodenum, or its intramural part, the ampulla, are often referred to as ampullary tumours. Adenocarcinomas in this location can be well circumscribed or very infiltrative, but a tumour centre in the ampulla is microscopically often obvious.

The rate of resectability of ampullary adenocarcinoma is reported to be 82-89% (56, 171, 172), which explains how this uncommon tumour can constitute a substantial fraction of resected periampullary adenocarcinoma.

A semantic aspect of the concept of “periampullary” is that a small ampullary tumour does not need to have any periampullary extension. That view does however not help communicating the pathologic findings, and most authors regard all ampullary tumours as belonging to the periampullary group.

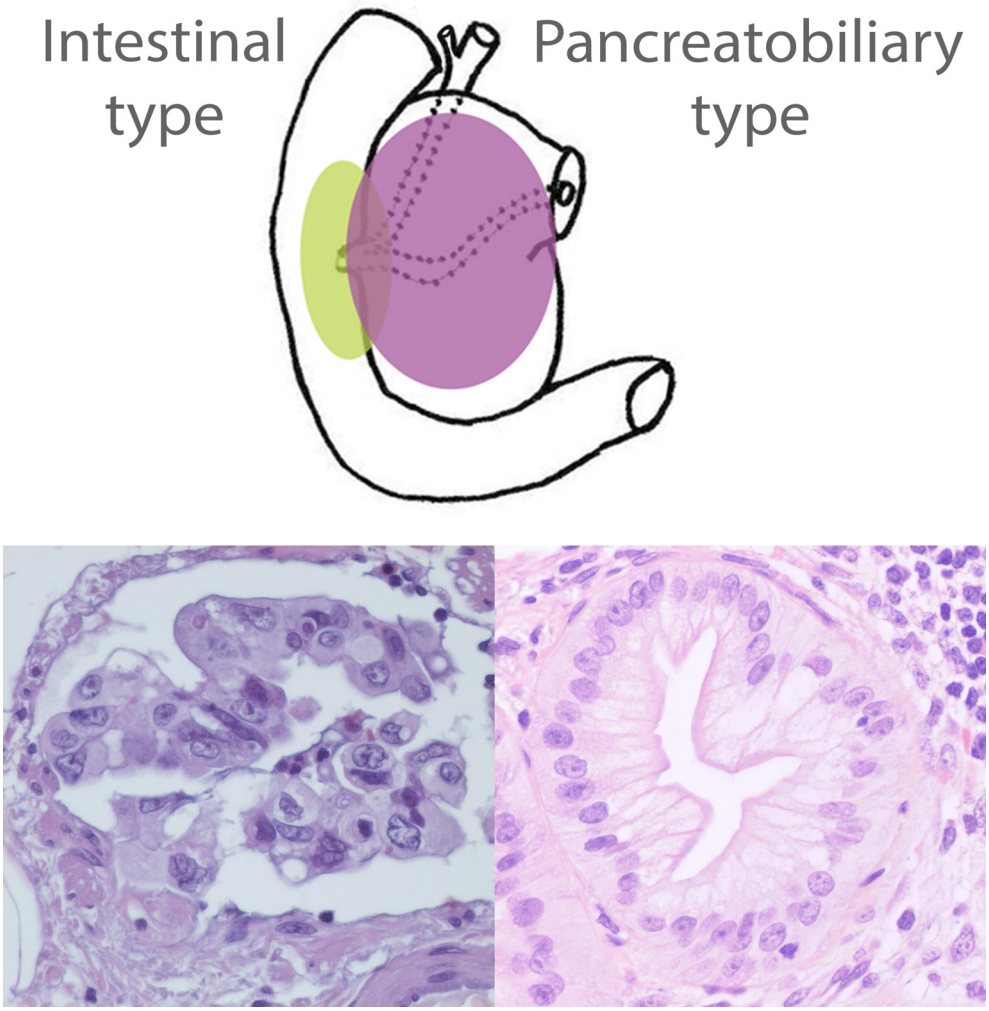

Figure 1
Distribution and examples of morphology of I-type and PB-type periampullary adenocarcinoma.

Duodenal adenocarcinoma

Small bowel adenocarcinoma is uncommon, and 50-75% is located in the duodenum (45-47). Approximately 75% of duodenal adenocarcinomas are located in the descending part of the duodenum (59), and a large fraction of small bowel adenocarcinoma is thus periampullary. The possibilities of a preoperative endoscopic assessment of duodenal adenocarcinomas often make the preoperative diagnosis clear. From a histologic and anatomic viewpoint an exophytic ampullary adenocarcinoma can look very similar to its duodenal counterpart, and assessing

tumour centre is the reasonable way of deciding on tumour origin. Resectability rates of duodenal adenocarcinoma are reported around 65% (59, 173).

Pancreatobiliary and intestinal morphological subtypes

During the last years, the distinction between PB- and I-type morphology in periampullary adenocarcinoma has evolved from a histological curiosity to a parameter with prognostic relevance (92), that also reflects tumours with different molecular profiles (91, 174). The anatomical basis for different types of ampullary adenocarcinoma was outlined in 1913 by Outerbridge (175) and the two basic morphologies, with different prognosis, were described by Kimura in 1994 (176). Since then, a shorter survival in PB-type tumours, as compared with I-type, has been described in several cohorts of either ampullary or periampullary adenocarcinoma (92, 177-181). The need for a morphological sub-classification of periampullary adenocarcinoma is most evident in ampullary tumours, where both histologic types are common (Figure 1). I-type morphology, with a corresponding better prognosis, has however also been described in distal bile duct and pancreatic adenocarcinoma (177, 182). The microscopic assessment of histologic type can be difficult and inter-observer agreement can be poor, especially when introducing a third category (183), often called “mixed” or “other”. Inter-observer variability can also be suspected from the varying proportions of tumour types in ampullary adenocarcinoma (92, 137, 176-181, 184-188) (Table 1), while larger series on the full spectrum of periampullary adenocarcinoma report 20-37% I-type and 49-63% PB-type (92, 177, 178). Despite the difficulties in assessing morphological type, this dichotomisation has been a major step in the field of periampullary adenocarcinoma, and it has been proposed that morphological type, rather than anatomical centre, should be the basis for classification (179).

Tumour stage (T-stage)

Tumour stage describes how advanced a tumour is locally, with T1 being the least advanced stage and T4 being the most advanced stage. The classification schemes of periampullary adenocarcinoma differ depending on tumour origin. A duodenal cancer invading the pancreas equals T4, while an ampullary counterpart with pancreatic invasion equals T3. Ampullary adenocarcinomas invading peripancreatic fat are classified as T4, while invasion of peripancreatic fat does not alter T-stage for distal bile duct and pancreatic cancer (189).

Shorter survival with increasing T-stage has been reported in cohorts of periampullary adenocarcinoma (177), distal bile duct adenocarcinoma (190), ampullary adenocarcinoma (179, 180), and pancreatic adenocarcinoma (191).

Table 1.
Morphological type in ampullary adenocarcinoma

Author, year	n	PB-type	I-type	Mixed/Other
Kimura, 1994	53	72%	25%	4%
De Pavia Haddad, 2010	97	48%	44%	7%
Kumari, 2013	91	47%	38%	14%
Carter, 2008	118	45%	46%	9%
Romiti, 2012	19	42%	58%	-
Matsubayashi, 1999	52	42%	58%	-
Westgaard, 2013	61	39%	61%	-
Bronsert, 2013	40	37.5%	45%	17.5%
Perrone, 2010	41	34%	49%	17%
McCarthy 2003	165	31%	57%	12%
Lowe, 2009	45	31%	29%	40%
Elebro, 2014 (paper I)	70	27%	73%	-
Baumhoer, 2008	175	24%	49%	27%

For cohorts with all periampullary tumour origins, only ampullary adenocarcinoma is shown.

Lymph node involvement (N-stage)

For adenocarcinomas of the pancreas, distal bile duct and ampulla, regional lymph node metastases are classified as either present (N1) or absent (N0), whereas lymph node metastases from duodenal adenocarcinoma are classified similarly to colorectal cancer, with 1-3 regional lymph node metastases (N1) being differentiated from 4 or more (N2) (189).

Presence of lymph node metastases is often reported as an independent factor of poor prognosis in cohorts of periampullary (55, 89, 92, 169, 177, 182, 192-196), pancreatic (191, 197), distal bile duct (58, 190, 198), and ampullary adenocarcinoma (179, 180, 199).

Survival in resected N0 periampullary adenocarcinoma is better for cases with a higher number of investigated lymph nodes, up to around 20 lymph nodes, and 10-15 has been suggested as a minimum requirement to accurately reflect N0-disease in the full spectrum of periampullary adenocarcinoma (200), and in pancreatic adenocarcinoma (201). In pancreatic cancer, a ratio between the number of involved lymph nodes and the total number of lymph nodes (lymph node ratio) can give more prognostic information than N-stage alone (202, 203).

Tumour size

Larger tumour size has been associated with shorter survival in cohorts of periampullary (55, 92, 182), or pancreatic cancer (197, 203).

Differentiation grade

Pancreatic cancer often has areas with different microscopic appearance, and differentiation grade is based on the poorest available differentiation grade (204).

A very large register study on resected pancreatic cancer (n=12,101) reported 36% with poor differentiation, and no association to survival in multivariable analysis (HR 1.01, 95% CI 0.90-1.13) (191), whereas several other studies on either periampullary, pancreatic, distal bile duct or ampullary adenocarcinoma found a shorter survival in cases with poor differentiation (58, 179, 194-196, 199, 203, 205). In another very large register based cohort on resected pancreatic cancer (n=7,086), with 37% poorly differentiated tumours, grade gave additional prognostic information in all stages and influenced survival more than lymph node metastases or tumour size (206).

Invasion of lymphatic vessels

Invasion of lymphatic vessels has been predictive of survival in ampullary adenocarcinoma (179, 180, 199), and in periampullary adenocarcinoma (177).

Invasion of microscopic blood vessels

Several reports on microscopic blood vessel invasion and prognosis after resection for pancreatic adenocarcinoma have found a shorter survival in cases with blood vessel invasion, reviewed in (207). A shorter survival has also been demonstrated in resected ampullary adenocarcinoma (199), and in cohorts of periampullary adenocarcinoma (92, 177, 182).

Perineural growth

Several reports on perineural invasion and prognosis after resection for pancreatic adenocarcinoma have found a shorter survival in cases with perineural invasion, reviewed in (207). Also in resected ampullary adenocarcinoma, survival has been shorter in cases with perineural tumour growth (179, 208).

Invasion of peripancreatic fat

Invasion in peripancreatic fat is an important factor in ampullary adenocarcinoma, since it raises the tumour stage to T4, but this parameter is seldom analysed as a separate parameter. In one study on pancreatic adenocarcinoma however, invasion of peripancreatic fat was an independent factor for shorter OS (209).

Margins

Local recurrence has been found to occur in around 80% of patients resected for pancreatic (210-212) or periampullary cancer (172), which indicates residual local disease after resection, and also suggests that tumour growth can be found in or close to margins in the specimen in a comparable fraction of cases. The reported fraction of cases with microscopically involved margins have however often been substantially lower, and sometimes as low as 15% in pancreatic cancer (191), and 0 mm is likely the threshold between involved and uninvolved margins in these series. Although this very low fraction of involved margins was prognostic for survival in a large register based cohort, the survival did not differ from cases with macroscopically involved margins. A more powerful prediction of prognosis has been reported in series making a thorough investigation of margins using 1 mm as a cut-off for involved margins (213, 214). It is noteworthy that the fraction of pancreatic or PB-type tumours with involved margins in these cohorts is close to 80%, and thus very similar to the incidence of local recurrence after resection.

Investigative prognostic and predictive biomarkers

SATB1

Special AT-rich sequence-binding protein 1 (SATB1) organizes the genome by regulating region-specific epigenetic modifications, and thus controls the expression of a large number of genes (215-217).

SATB1-expression has been demonstrated to confer a more aggressive tumour phenotype and a shorter patient survival in several cancer forms, e.g. breast cancer (215), prostate cancer (218), laryngeal squamous cell carcinoma (219), nasopharyngeal cancer (220), hepatocellular carcinoma (221), rectal cancer (222), cutaneous malignant melanoma (223), epithelial ovarian cancer (224), glioma (225) and gastric/oesophageal adenocarcinoma (226, 227).

Before paper II (228), there were no publications on the expression and prognostic correlates of SATB1 in any of the periampullary adenocarcinoma types.

SATB2

SATB2 (Special AT-rich sequence-binding protein 2), a close homologue to SATB1, is involved in osteoblast differentiation and craniofacial patterning (68, 69), and has been demonstrated to be abundantly expressed in normal colorectal mucosa and colorectal adenocarcinomas, but more sparsely in other types of carcinomas (70). Low or absent SATB2-expression has further been shown to be a marker of malignant behaviour and poor prognosis in colorectal cancer (71, 72), whereas high expression correlated to a better response to neoadjuvant chemotherapy in rectal cancer and neoadjuvant/adjuvant chemotherapy in stage III-IV colorectal cancer (73).

Before paper II, the expression of SATB2 had been described to be negative in all of 25 examined pancreatic adenocarcinomas and in all but one of 15 examined bile duct adenocarcinomas in a large screening study on normal and cancerous human tissue (229). However, the expression of SATB2 had not been described in ampullary or duodenal adenocarcinoma.

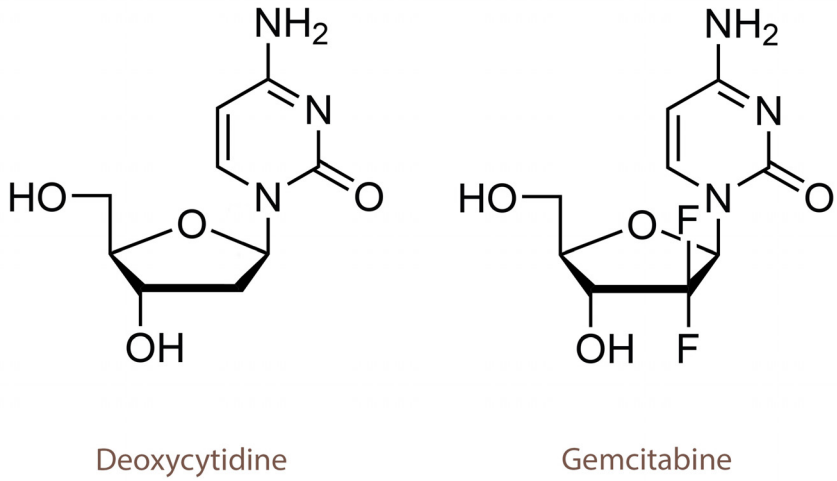

Figure 2
Deoxycytidine and gemcitabine. This figure consists entirely of information that is common property and contains no original authorship. No copyright.

hENT1

Human equilibrative nucleoside transporter 1 (hENT1) provides the major route for nucleosides and gemcitabine (Figure 2) to enter a cell, and is one of the most extensively studied biomarkers in the context of gemcitabine response in pancreatic cancer (Figure 3). High expression of hENT1 has been predictive of gemcitabine response in large cohorts and meta-analyses of pancreatic cancer (230-232), but has been little studied in other periampullary adenocarcinoma. In a small cohort of patients with ampullary adenocarcinoma, who did not receive adjuvant chemotherapy, hENT1 expression was found to be higher in I-type than in PB-type tumours (186), and to be associated with a shorter OS (233).

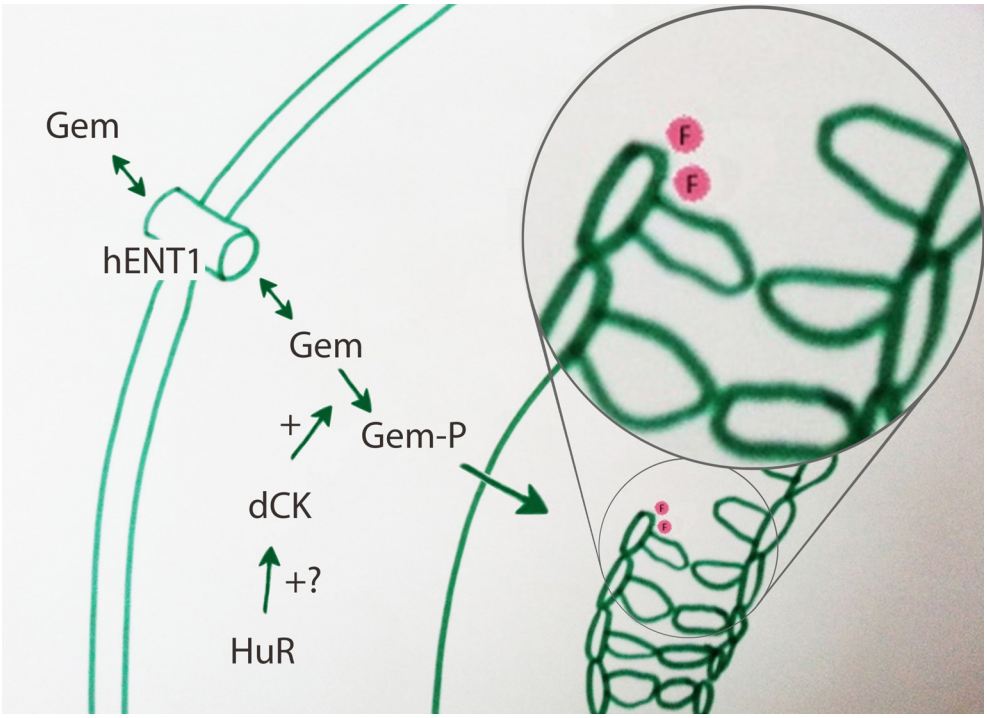

Figure 3
Gemcitabine metabolism

dCK

Deoxycytidine kinase (dCK) is a deoxyribonucleoside kinase that by phosphorylating deoxyribonucleosides enables their incorporation into DNA, thereby providing an alternative to *de novo* synthesis of DNA precursors. Gemcitabine is a nucleoside analogue in which two hydrogen atoms are replaced by fluorine atoms, and phosphorylation by dCK is a rate-limiting and required step before incorporation of gemcitabine into DNA, and subsequent masked chain termination and apoptosis (234). Expression of dCK is required for gemcitabine sensitivity and cell lines with induced resistance show decreased dCK RNA levels, while influx of gemcitabine into the cells is unaffected (235). High dCK expression has been associated with longer OS in patients treated with adjuvant gemcitabine, but not in untreated patients (231), and a meta-analysis found that either high protein or gene expression of dCK predicted a longer OS and RFS in gemcitabine treated patients with pancreatic cancer (232). However, another study on pancreatic cancer found an association between dCK protein expression and better response to 5-FU, but not to gemcitabine (236).

Before paper III (237), there were no publications on the prognostic or predictive value of dCK in I-type periampullary adenocarcinoma, even though many of these patients receive adjuvant gemcitabine or 5-FU.

HuR

Human antigen R (HuR) is an RNA-binding protein that performs posttranscriptional regulation of several proteins in response to stress or growth signals, thereby stabilizing mRNAs related to proliferation, angiogenesis and evasion of apoptosis (238, 239). Cytoplasmic HuR is also increased in malignant cells as compared with corresponding normal cells, and has been found to be associated with adverse clinicopathological factors and a shorter OS in several different cancer forms (240), e.g. gastric cancer (241), gallbladder cancer (242), breast cancer (243), urothelial cancer (244) and non-small cell lung cancer (245). In pancreatic cancer, however, two small studies found high HuR expression to be associated with a longer OS in patients treated with gemcitabine, and HuR was also demonstrated to bind dCK-mRNA, which might explain a greater sensitivity to gemcitabine in tumours with high levels of HuR (246, 247). Low nuclear HuR expression has not been associated with prognosis or prediction of response to chemotherapy, but a high cytoplasmic to nuclear ratio of HuR has been associated with a shorter OS colorectal cancer (248).

Before paper III, there were no reports on the expression and prognostic correlates of HuR in I-type periampullary adenocarcinoma.

EGFR, HER2 and HER3

Members of the HER (human epithelial growth factor receptor) family of tyrosine kinase receptors are essential for human development and growth, and they are overexpressed in several human cancers. They consist of four closely related transmembranous molecules, EGFR (HER1), HER2, HER3 and HER4. There are several HER-ligands, including epidermal growth factor (EGF), transforming growth factor-a (TGF-a), and neuregulins (249). The affinity for HER-ligands differs between members of the family, and ligand binding causes hetero- or homodimerization of receptors and intracellular transphosphorylation, which activates several intracellular signalling-cascades important for cell survival,

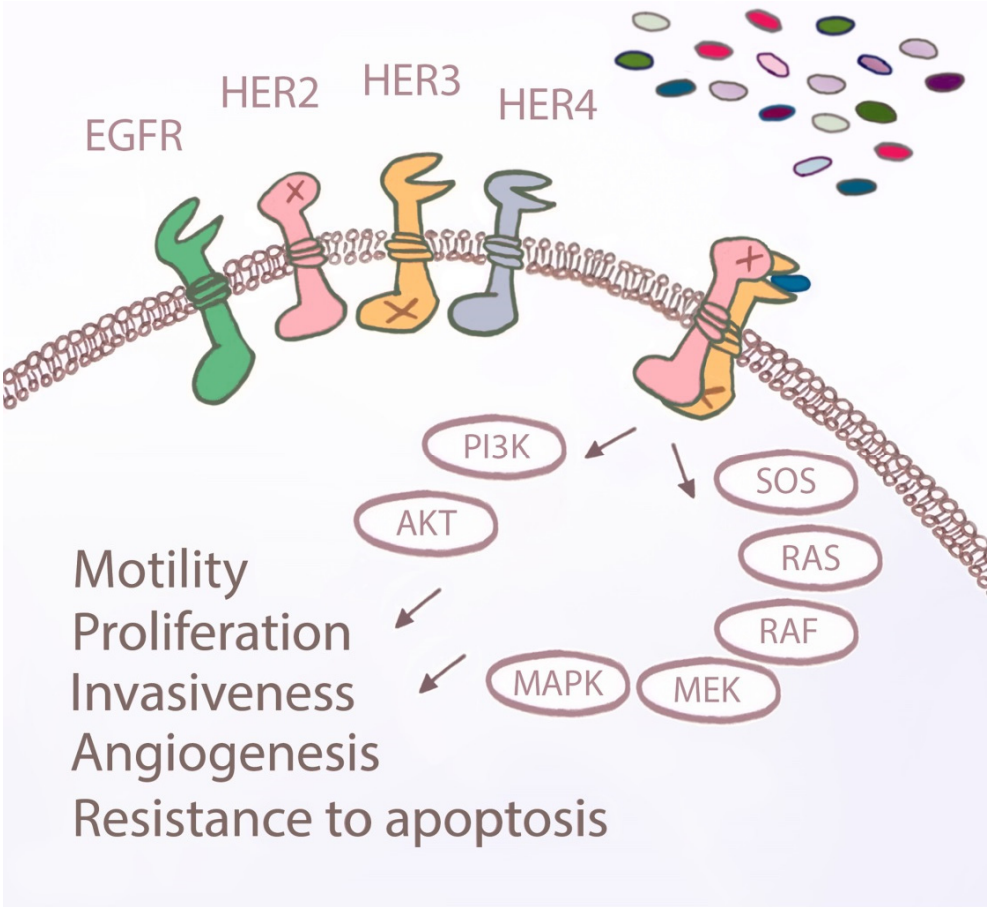

Figure 4
EGFR, HER2, HER3 and HER4 dimerization and signalling.

proliferation and growth. Combinations of HERs give dimers that vary in stability, affinity for their ligands and activation of different signalling cascades (250) (Figure 4).

EGFR

EGFR is often highly expressed in pancreatic cancer, and was associated with worse prognosis in a meta-analysis on pancreatic cancer (251), although several studies did not find any prognostic effect of EGFR expression on OS (252-254). EGFR expression has also been shown to be more common in PB-type than in I-type ampullary adenocarcinoma (255), and overexpression has been associated with shorter OS in I-type but not in PB-type tumours (256).

There are several drugs, targeting either the extracellular domains or the intracellular tyrosine kinase domains of the HERs, that give survival benefits in selected cases of breast, colon, gastric and lung cancer (250), and combinations of HER-active drugs have been shown to further improve survival compared with single HER-therapy (257). Addition of the EGFR tyrosine kinase inhibitor erlotinib to gemcitabine led to an increased OS in a study on patients with advanced pancreatic cancer (101), the improvement was however modest and erlotinib is therefore rarely used for pancreatic cancer in clinical practice. Other EGFR active drugs have not led to a prolonged OS, when added to standard chemotherapy (250).

HER2

The reported rates of HER2 overexpression in pancreatic cancer, defined as 3+ in IHC staining or gene amplification by in situ hybridization (ISH), vary from 0%-11% (258-265). Similarly to other tumour types, high expression of HER2 in pancreatic cancer has been associated with a shorter survival (266), but other studies have found the opposite (267). Addition of trastuzumab to gemcitabine in metastatic pancreatic cancer overexpressing HER2 (2+ or 3+) gave no clear survival benefit compared with the expected survival upon gemcitabine alone (268).

In tumours of the ampulla, distal bile duct and gall bladder, the frequency of HER2 overexpression has been low, and comparable to pancreatic cancer in a few small studies (258, 259, 265), whereas larger studies have found overexpression in 6-13% of ampullary tumours (188, 269), and in 23% and 17% of tumours of the bile duct and gall bladder (270). There is a single case report on HER-targeted treatment in advanced ampullary adenocarcinoma with HER2 overexpression (271), but to date not yet any larger series or trials.

HER3

HER3 has a deficient intracellular kinase domain, but heterodimerization with other HERs leads to intracellular survival signals (250). Oncogenic *HER3* mutations are found in 11% of colorectal and gastric cancer, but less often in other types of cancer, and leads to oncogenic signalling when expressed together with HER2 (272). HER3 mutations have also been found in 11% of ampullary adenocarcinoma, equally distributed between PB- and I-type tumours (91).

The present investigation

Aims

The main objective of this thesis (paper I-IV) was to create and characterize a cohort of periampullary adenocarcinoma, and to study potentially prognostic or predictive biomarkers using tissue microarrays (TMA).

Specific aims

- To assess if a standardized method of sectioning pancreatoduodenectomy specimens affects decision on tumour origin and margin status, and to assess how blind revisions of slides affect these parameters.
- To generate a consecutive study cohort of resected periampullary adenocarcinomas, with detailed information on histopathological parameters, neoadjuvant, adjuvant and palliative oncological treatments, recurrences and death.
- To build a TMA, with tumour material from all primary tumours and selected lymph node metastases, from the cohort.
- To examine the expression and prognostic implications of SATB1 and SATB2 in the cohort, with reference to morphological tumour type.
- To examine the expression and prognostic implications of hENT1, HuR and dCK in the cohort, with reference to morphological tumour type.
- To examine the expression and prognostic implications of EGFR, HER2 and HER3 in the cohort, with reference to morphological tumour type.

Material and Methods

Date of surgery and data on the original pathology report were obtained from the pathology reports.

All haematoxylin & eosin slides were revised in a blinded manner. When present, slides with IHC stains were not revised.

Data on neoadjuvant therapy, adjuvant therapy, date and place of first recurrence and date of death were obtained from patient records. Data on survival, and date of death were also gathered from the Swedish National Civil Register.

Paraffin blocks with TMAs were constructed using three 1mm cores from each primary tumour, from at least two different blocks of formalin fixed paraffin embedded tissue. Up to three 1 mm cores were also obtained from paired lymph node metastases (figure 5).

IHC stains were performed on 4 micrometre thick sections of the constructed TMA-blocks.

Different models of assessing the IHC stainings were used for different antibodies in paper II-IV, to assess the expression of the proteins of interest.

All statistical calculations were performed using SPSS. For comparisons of levels of annotated expression of one protein between tumour types or between primary tumours and metastases, t-tests were done. To assess associations between the expression of different proteins and other parameters, Chi-square-tests were done. To evaluate the associations between annotated expression of a protein and survival, Kaplan Meier and log-rank analyses were performed. To confirm differences in survival between groups, Cox regression analyses were done, both without taking the distribution of other factors into account (univariable analysis) and taking into account the distribution of selected parameters that may affect survival (multivariable analysis). In cases where survival seemed to depend on combinations of a certain protein expression and adjuvant chemotherapy, the interaction was assessed using constructs of protein expression (+/-) x adjuvant chemotherapy (+/-) in relation to survival, using univariable Cox regression analysis.

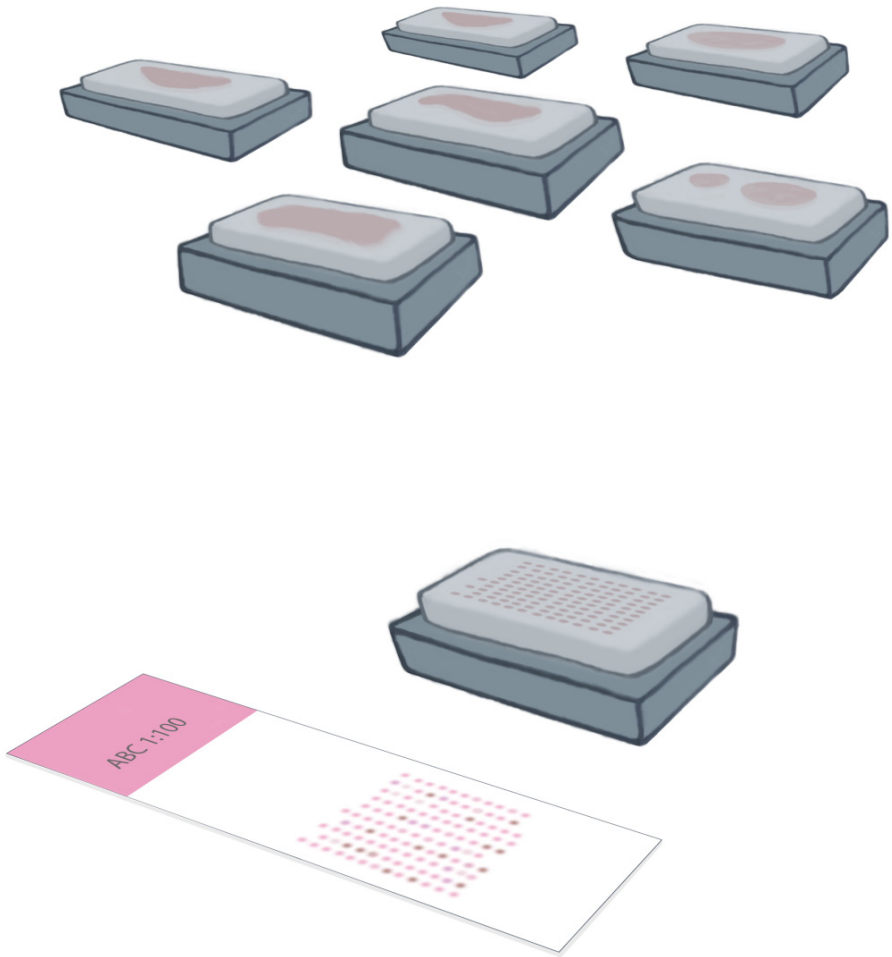

Figure 5
Construction of a tissue microarray. Cores from several donor blocks with paraffin embedded tissue are arranged in a matrix in a recipient paraffin block, from which multiple sections can be made.

Summary of Results and Discussion

Paper I

Post re-evaluation of tumour origin showed a significantly higher proportion of distal bile duct origin (39% vs 21%) and a lower proportion of ampullary origin (26% vs 45%) in the standardized group as compared with the non-standardized. Tumour origin is fundamental for which treatment protocol a patient can enter and also has prognostic implications (167). It has also been shown previously that the morphological distinction between PB- and I-type morphology has prognostic implications, not only in ampullary adenocarcinomas, but in all periampullary adenocarcinomas, regardless of tumour origin (182). Moreover, while differences in the expression of cytokeratins and mucins according to morphology have been observed in ampullary carcinomas (273), these differences seem to be less evident in series stratified solely by the anatomical centre of the ampullary adenocarcinomas (274). These findings suggest that morphological and molecular tumour characteristics have a greater prognostic impact than the appreciated tumour origin.

The re-evaluated number of involved lymph nodes, found by the pathologist in the specimen, was significantly higher in the standardized group, but the proportion of cases with involved lymph nodes (N1) did not differ significantly between the standardized and the non-standardized group (72% vs 57%). Re-evaluation of slides revealed N1 in 20% (14/70) of non-standardized cases assigned as N0 in the original report, and the fraction of non-standardized cases with N1 rose from 46% to 57%. Previous reports on non-standardized series of resected periampullary adenocarcinoma often describe less than 60% N1 (192-196, 275, 276), while our standardized group and other standardized series describe over 70% N1 (213, 214).

Our standardized group had a significantly larger proportion of cases with involved margins, as compared with the non-standardized group. Re-evaluation of slides also increased the number of cases with involved margins in the non-standardized group. Re-evaluations also rendered a large group of non-standardized cases where margin status could not be fully assessed, due to sparse sampling of margins. OS was significantly longer in the re-evaluated group with

uninvolved margins as compared with the cases that had uninvolved margins according to the original report.

Our results thus show that a standardized protocol can affect assessed tumour origin, increase the number of involved lymph nodes and increase the fraction of cases with involved margins. Re-evaluations of margin status can increase the prognostic value of uninvolved margins.

Paper II

In PB-type tumours, 15% of primary tumours and 17% of metastases expressed SATB1, and 20% were positive in either the primary tumour or in a metastasis. In I-type tumours, 25% were positive in either the primary tumour or in a metastasis. There was a significant association between gemcitabine based adjuvant chemotherapy and tumour origin within the PB-type tumours, and between adjuvant chemotherapy and involved lymph nodes within the I-type tumours. Except for these two factors, the distribution of patient and tumour characteristics did not differ significantly between patients who had received or not received adjuvant chemotherapy in neither of the histological subtypes.

In PB-type tumours, SATB1 expression was prognostic for a shorter RFS and OS, and significance was retained in multivariable analysis for OS. When stratifying for adjuvant gemcitabine, SATB1-positive cases receiving adjuvant gemcitabine had a prolonged OS, compared with SATB1-positive cases not receiving adjuvant gemcitabine, while there was no significant difference in OS between SATB1-negative cases receiving or not receiving adjuvant gemcitabine. The interaction between SATB1 and adjuvant gemcitabine in relation to OS approached significance ($p_{interaction}$=0.066).

In contrast to the PB-group, SATB1 expression was not prognostic for OS or RFS in the full group of I-type tumours. When stratifying for adjuvant chemotherapy there was no significant difference in RFS or OS between SATB1-negative cases receiving or not receiving adjuvant chemotherapy, whereas SATB1-positive cases had a prolonged RFS, and a tendency towards a prolonged OS, after adjuvant chemotherapy, compared with SATB1-positive cases not receiving adjuvant chemotherapy. The interaction between SATB1 and adjuvant chemotherapy was significant in relation to RFS ($p_{interaction}$=0.021).

Our finding of an association between SATB1 expression and poor prognosis in PB-type tumours is in line with previous reports on several types of cancer. A potential treatment predictive effect of SATB1 expression, seen in both PB- and I-type tumours, has however not been described before, and merits confirmation, both in a mechanistic context and in additional patient cohorts.

SATB2 expression was present in 3% of PB-type tumours, and there were no significant associations with clinicopathological parameters. Since SATB2-expression was only seen in 3 out of 107 PB-type tumours, statistical analyses on survival are hazardous to interpret. However, a significantly shorter OS and RFS were observed for SATB2-positive cases, and this significance was retained in multivariable analysis for both OS and RFS.

In I-type tumours, SATB2 expression was positive in 13% of cases, and there was a significant association with growth in peripancreatic fat, but not with any other clinicopathological factor. Expression of SATB2 was not prognostic, neither for OS nor RFS, and there were no significant differences in survival between SATB2-positive cases receiving or not receiving adjuvant chemotherapy, but, of note, there were no recurrences or fatalities among SATB2-positive I-type cases receiving adjuvant chemotherapy. This observation may suggest a similar treatment predictive function for SATB2 in I-type tumours as observed for SATB1 and would also be in line with a previously described treatment predictive function of SATB2 in colorectal adenocarcinoma (277).

Paper III

There was a higher expression of hENT1 and HuR, and a higher HuR C/N ratio in I-type as compared with PB-type primary tumours, but no difference in expression of dCK. There were no associations between the expression of dCK and HuR.

In PB-type tumours, hENT1 expression was inversely associated with poor differentiation and dCK expression was not associated with any other parameter. There were no associations between expression of hENT1 or dCK and survival, which contrasts previous results in large cohorts on the response predictive value of high expression of hENT1 or dCK.

A high HuR C/N ratio was associated with male sex, HuR expression and positive or unassessable margins (R1-Rx vs. R0). HuR C/N ratio was not prognostic in the full group of PB-cases, or in the subgroup that had not received adjuvant gemcitabine. However, in patients that received adjuvant gemcitabine, a high HuR C/N ratio was significantly associated with a reduced OS, and there was a significant interaction between protein expression and adjuvant gemcitabine in relation to OS, ($p_{interaction}$=0.028), and a borderline significant interaction in relation to RFS, ($p_{interaction}$=0.053). This finding contrasts previous reports on two small series of gemcitabine-treated patients with pancreatic cancer, where high HuR expression was found to be associated with a prolonged survival (246, 247). The finding is plausible, however, as HuR increases the expression of proteins related to proliferation, angiogenesis and evasion of apoptosis, and thus promotes a more

malignant phenotype. Our findings of an association between a high HuR C/N ratio and worse prognosis also harmonize with a majority of reports on HuR in other tumour types (240).

In I-type tumours, hENT1 expression was associated with HuR expression, duodenal origin and larger tumour size, and inversely associated with growth in lymphatic vessels. High hENT1 expression was an independent prognostic factor for longer RFS. This finding contrasts previous reports on ampullary and gastric cancer, where hENT1 expression was demonstrated to be associated with a shorter survival (233, 278).

Expression of dCK was significantly associated with a higher proportion of uninvolved margins, but not with survival in the full group of I-type tumours, or in the adjuvant untreated subgroup. In the subgroup that received adjuvant therapy, however, high dCK expression was significantly associated with a prolonged RFS and the treatment interaction was significant ($p_{interaction}$=0.023). The corresponding analysis could not be done for OS, as there were no fatalities among the nine patients with high dCK expression having received adjuvant chemotherapy. Several of the patients with I-type tumours had received adjuvant gemcitabine, but there are indications that dCK also increases sensitivity to 5-FU (236). A potential response predictive effect of dCK has not been described in this tumour type before, and merits further study.

HuR expression was inversely associated with perineural growth. High expression was associated with a significantly longer OS in the full group of patients with I-type tumours, but significance was not retained in multivariable analysis. This finding is somewhat surprising, and differs from reports on several other tumour types and the concept of HuR as a positive regulator of malignant behaviour (240). Our finding may however well be coincidental, as suggested by the inverse association with perineural growth, together with non-significance in multivariable analysis.

There were no significant associations between HuR C/N ratio and any clinicopathological parameter, apart from HuR, in I-type tumours. A high HuR C/N ratio was significantly and independently associated with a longer OS and borderline significantly associated with a prolonged RFS.

Paper IV

HER2 and HER3 were more often strongly expressed in I-type, as compared with PB-type, periampullary adenocarcinoma. There were no HER2 3+ cases among the PB-type tumours, compared with 6% in I-type tumours, and all HER2 3+ cases were of ampullary tumour origin. Evaluable SISH confirmed that all 3+ cases had a *HER2* gene amplification, and, in addition, one 2+ case of PB-type ampullary adenocarcinoma was *HER2* amplified. In total, HER2 overexpression (IHC 3+ or SISH+) was found in 7% of ampullary adenocarcinoma (and in 8% of I-type ampullary adenocarcinoma).

In PB-type tumours, there were no associations between high expression of HERs and clinicopathological parameters. In I-type tumours, high EGFR expression was associated with larger tumour size and high HER3 expression was inversely associated with tumour stage, perineural growth, blood vessel invasion, growth in peripancreatic fat and recurrence.

In I-type tumours, expression of EGFR was significantly associated with shorter RFS and OS, and HER3 expression was significantly associated with longer RFS, but these associations were not independent of other prognostic factors.

In PB-type tumours, high EGFR expression was not prognostic in the full group. However, in strata of patients that had either received or not received adjuvant gemcitabine based chemotherapy, EGFR expression had prognostic implications. There was a significantly shorter RFS and OS in gemcitabine treated patients with high tumour-specific EGFR expression, as compared with gemcitabine treated patients with low tumour-specific EGFR expression. Correspondingly, a longer OS after adjuvant gemcitabine was seen in cases with low EGFR expression, but not in cases with high EGFR expression. In PB-type tumours, the interaction between EGFR expression and adjuvant gemcitabine was significant in relation to OS ($p_{interaction}$=0.042). HER3 expression was not prognostic in PB-type tumours.

The potentially predictive effect of EGFR expression in PB-type periampullary adenocarcinoma has not been described before, and should be interpreted with caution due to the retrospective exploratory nature of the cohort and the investigation, and since the risk for type I errors is not negligible after performing multiple tests.

The finding of HER2 overexpression in 7% of ampullary adenocarcinoma is in line with previous reports (188, 269), and may become clinically useful as adjuvant and palliative treatments become more targeted and individualized. A major obstacle, however, is that I-type periampullary adenocarcinomas are uncommon, which makes clinical trials challenging.

Limitations to the study (paper I-IV)

One limitation that is evident in paper I is that the revision of slides could lead to a biased evaluation, even though it was performed in a blinded manner. In order to evaluate the histopathology parameters, all slides from a case needed to be assessed, and it was sometimes obvious that some cases were not part of the standardized protocol. In paper I, the finding of invasion in blood vessels was significantly more common in the non-standardized group, which may be a consequence of an unintentionally more thorough search for evaluable parameters in cases that offered limited possibilities of assessing other parameters.

All four papers share another important limitation in that many tests have been made, which increases the risk for type I errors, i.e. detection of significances that are coincidental. It is well known that the likelihood of getting a significant p-value increases when multiple tests are made, as compared with a situation when a few targeted tests are made. This "seek, and ye shall find" problem has, however, no simple solution. The easiest way to adjust the threshold for significance is the Bonferroni correction method, which divides the selected p-value threshold (often 0.05) by the number of tests made. This method has however been criticised for being too consevative and thus increasing the risk for type II errors. The nature of the studies in paper I-IV is exploratory, rather than confirmatory, and as such would loose value if the risk for type II errors increases too much.

The question of representativity can always be raised when TMAs are used. Biomarkers that are heterogenously expressed in a tumour can be missed in analyses based on this method, compared with the use of full face sections, but known examples of this problem are few (279). Another possible problem with TMAs is that the included tissue cores may vary regarding fixation time in formalin and age. In paper IV, SISH for *HER2* failed in approximately 50% of cases that had 2+ or 3+ expression of HER2 IHC. Prolonged fixation time is a well known source of ISH-failure, and was probably the case in paper IV. Although IHC is less sensitve to fixation time, it cannot be disregarded as a possible sourse of error. The annoted expressions of biomarkers in paper II-IV showed no significant associations with year of surgery (i.e. age of the sample), but we have no data on fixation time in formalin for the tumours in the cohort.

One limitation to the cohort is that the I-type group had relatively few events during the follow up period, which limits the possibilities to make adjustments in multivariable Cox regression analysis, especially when the material is stratified for both biomarker expression and adjuvant therapy. We have however chosen to adjust for known prognostic parameters, rather than only adjusting for one or two factors. One interesting aspect of this problem was seen during the preparation of paper IV, where high EGFR expression in I-type tumours was found to be

associated with a shorter OS. The survival difference was non-significant in multivariable analysis when adjusting for the parameters we knew were prognostic in our cohort, but significant when adjusting for only the most well-known parameters, i.e. T-stage, N-stage, tumour size and grade.

Conclusions and Future Perspectives

Both method of sectioning and microscopic assessment affect important pathology parameters in resected periampullary adenocarcinoma.

Several of the investigated proteins have prognostic implications in resected periampullary adenocarcinoma, and for a few there were indications of a potentially response predictive effect. In several instances, the prognostic effect of a studied protein differed between PB- and I-type periampullary adenocarcinoma.

A few of the findings in paper II-IV merit further study. The findings on SATB1 and dCK in I-type tumours, and EGFR in PB-type tumours, are novel and indicate a potentially response predictive effect. This could become clinically relevant if it can be reproduced in other cohorts and the mechanistic foundation can be mapped.

From the presented studies (paper II-IV), it is evident that 65 I-type tumours is a barely sufficient number to study potential response prediction in relation to the dichotomized expression of a biomarker, since groups become very small. I-type periampullary adenocarcinoma is rather uncommon and prospective studies would thus require cooperation between several centres.

Another future direction for these tumour types is the increasing use of sequencing of multiple genes (massive parallel sequencing/next generation sequencing). It is reasonable to assume that this methodology will be used in clinical practice for all highly lethal malignancies within years. A development in that direction would not only generate enormous amounts of data, but also a vast opportunity to find gene alterations that can cluster and stratify patients according to prognosis and expected response to specific treatments. In this context, the distinction between explorative dredging of data and confirmatory analyses will become increasingly important.

Populärvetenskaplig sammanfattning

Cancer i området där pankreas och gallvägar möter duodenum kallas periampullär cancer, vilket är ett användbart begrepp då man inte alltid vet exakt vilken vävnad tumören utgått från. Pankreascancer är den vanligaste periampullära cancern, med cirka 1000 nya fall/år i sverige, och de övriga periampullära tumörtyperna är tillsammans cirka hälften så vanliga. Överlevnaden hos patienter med pankreascancer är mycket låg, vilket gör att pankreascancer är den fjärde vanligaste orsaken till cancerdöd i västvärlden. Prognosen har, till skillnad från de flesta övriga cancertyper, inte förbättrats de senaste decennierna och om denna trend håller i sig kommer pankreascancer att bli en allt mer vanlig orsak till cancerdöd.

Avhandlingsarbetet har fyra delar, där den första (paper I) beskriver mikroskopiska fynd hos alla 175 patienter med periampullära tumörer som opererades i Lund och Malmö under perioden 2001-2011 och hur en mer nogrann undersökning av operationspreparaten ger mer information om prognostiska faktorer. Arbete 1 beskriver även hur en nogrann förnyad mikroskopisk bedömning kan ge bättre prognostisk information. De studerade tumörerna har fyra olika vävnadsursprung, men p.g.a. att bedömningen av tumörursprung varierar väldigt mellan olika studier och förefaller vara en ganska subjektiv bedömning, så har de studerade tumörerna delats in i två grupper utifrån mikroskopiskt utseende. Denna indelning gör att grupperna blir större och att delar av gränsdragningsproblematiken försvinner. Grupperna kallas pankreatobiliär och intestinal, vilket beskriver om det mikroskopiska utseendet påminner mest om tumörer från pankreas och gallvägar eller om tumörer från tarmen.

För studierna som presenteras i arbete 2-4 (paper II-IV) byggdes vävnadsmatriser med tumörvävnad från alla patienter, och från patientjournaler hämtades information om vilka onkologiska behandlingar patienterna fått och när de fick tumöråterfall. Från folkbokföringsregistret hämtades information om när de dog. På detta sätt gavs möjlighet att göra många riktade undersökningar av olika proteiner i tumörerna (med hjälp av s.k. immunohistokemisk analys), där utfallet kunde jämföras med tid till tumöråterfall och död, även i relation till den givna onkologiska behandlingen.

I artikel 2 (paper II) beskrivs de två närbesläktade proteinerna SATB1 och SATB2 i tumörerna. Patienterna med pankreatobiliära tumörer som uttryckte SATB1 hade

en kortare tid till tumöråterfall och död, jämfört med pankreatobiliära tumörer som inte utryckte SATB1, men det sågs även en tendens till att tumörer med SATB1-positivitet samvarierade med högre känslighet för den vanligaste typen av cytostatika, gemcitabine. I gruppen intestinal typ sågs ingen prognostisk koppling, men uttryck av SATB1 hade en koppling till bättre svar på cytostatika. SATB2 uttrycktes bara i 3% av de pankreatobiliära tumörerna, vilket gör det svårt att dra några slutsatser. Dessa 3% hade dock en kortare tid till tumöråterfall och död, vilket gör att resultaten för SATB2 påminner om de för SATB1, i pankreatobiliär tumörtyp. SATB2 var inte prognostiskt i intestinal tumörtyp, men bland cytostatiskabehandlade fall med SATB2-uttryck sågs inga tumöråterfall eller dödsfall. Fallen var för få för att ge säkra resultat, men fynden påminner om SATB1 i intestinal tumörtyp.

I artikel 3 (paper III) beskrivs tre proteiner som är viktiga för hur celler hanterar upptaget av den i dessa tumörtyper vanligaste typen av cytostatika, gemcitabine. hENT1 är ett kanalprotein som behövs för att gemcitabine ska kunna komma in i celler. dCK modulerar gemcitabine med en fosfatgrupp, så att gemcitabine stannar i cellen och så att det kan byggas in i DNA och på så sätt skada cellen. Mängden av både hENT1 och dCK tros teoretiskt sett därför kunna påverka svar på behandling med gemcitabine. Det tredje proteinet, HuR, påverkar mänderna av många proteiner som påverkar en cancertumörs farlighet, men tros även kunna öka mängden dCK, vilket i så fall skulle kunna göra tumörerna mer känsliga för gemcitabine. Resultaten visade ingen koppling mellan mängden hENT1 och prognos eller svar på gemcitabine i den pankreatobiliära tumörgruppen. I den intestinala tumörgruppen sågs en längre tid till tumöråterfall för de patienter vars tumörer uttryckte mer hENT1, oavsett om cellgifter givits eller ej. I den pankreatobiliära tumörgruppen sågs ingen koppling mellan nivåer av dCK och prognos eller svar på gemcitabine. I den intestinala gruppen sågs ingen koppling mellan dCK-nivåer och prognos, men tumörer med mer dCK svarade bättre på cytostatika än de med lite dCK. För HuR i pankreatobiliär tumörtyp sågs ingen prognostisk effekt i hela gruppen, men en koppling mellan en högre kvot av mängden i cellernas cytoplasma och kärna (C/N-kvot) och kortare tid till tumöråterfall och död hos patienter som fått behandling med gemcitabine. I den intestinala tumörtypen sågs en prognostisk effekt, med längre tid till död, och en på gränsen till längre tid till tumöråterfall, hos dem med hög C/N-kvot.

Av fynden i artikel 3 är observationen att högt dCK tycks vara kopplat till en ökad känslighet för cellgifter i tumörer av intestinal typ mest spännande, då den inte beskrivits tidigare och då färgningen var relativt enkel att bedöma. Det är också önskvärt att identifiera biomarkörer som förutspår svar på behandling i denna tumörtyp, där man i nuläget inte vet säkert om cytostatika gör nytta, eller enbart ger biverkningar.

I artikel 4 (paper IV) beskrivs tre närbesläktade ytproteiner som förmedlar signaler från utsidan till insidan av cellerna; EGFR, HER2 och HER3. För HER2 beskrivs även mängden kopior av dess gen, för de tumörer som hade mycket av proteinet. Dessa proteiner är intressanta, dels för att de kan göra cancerceller farligare, men även för att det finns flera olika cancermediciner, s.k. målstyrda behandlingar, som attackerar just dessa proteiner. Resultaten visade en hög mängd av HER2-proteinet i 6% av de intestinala tumörerna och en korresponderande ökad mängd HER2-genkopior. Detta är intressant då dessa fynd indikerar att ett riktat läkemedel, med god biverkningsprofil, som är verksamt i andra tumörtyper, eventuellt kan vara ett behandlingsalternativ även i intestinal typ av periampullär cancer. Inga pankreatobiliära tumörer hade högt uttryck av HER2-proteinet, men en tumör visade en ökad mängd genkopior. Nivåer av HER2 hade ingen inverkan på prognos eller svar på cytostatiska, varken i pankreatobiliär eller intestinal tumörtyp. Nivåer av EGFR var inte prognostiskt i pankreatobiliär tumörtyp men hos patienter som behandlats med gemcitabine var högt EGFR kopplat till kortare tid till tumöråterfall och död. Det sågs ingen koppling mellan nivåer av HER3 och prognos, eller behandlingssvar, i pankreatobiliär tumörtyp. I intestinal tumörtyp sågs en sämre prognos för tumörer med högt uttryckte av EGFR, och en bättre prognos för tumörer med högt uttryck av HER3, men dessa samband var svagare än för andra prognostiska variabler, och därför mindre pålitliga.

En koppling mellan högt uttryck av EGFR och sämre svar på gemcitabine har inte beskrivits tidigare, och kan få klinisk betydelse om fynden kan bekräftas i andra studier.

Acknowledgements

Supervisor Karin Jirström; thank you for welcoming me into your generous and vibrant group, and for introducing me to the field of TMA and biomarkers. It has been an amazing journey! Supervisor Jakob Eberhard; thank you for your calm, yet precise, analyses, and for sharing your expertise in theoretical and practical oncology.

Past and present fellow PhD students in the research group; Björn, Alexander, Sakarias, Jenny, Liv, Anna, Karolina, Richard, Charlotta, Carl Fredrik, Jonna, Sebastian and Gustav; thank you for a great atmosphere and much fun. Special thanks to Björn for making the lab run smoothly, always with a smile on your face, and to Emelie for bringing functional expertise into the group.

Other co-authors; Mathias and Margareta, thank you for great collaborations.

Administrative staff at the Division of Oncology and Pathology; Susanne and Magnus, thank you for technical and administrative support.

Head of the Department of Clinical Sciences; Bo Baldetorp, thank you for a stimulating research environment.

Past and present colleagues at Clinical pathology in Lund and Malmö; thank you for sharing your insights on histopathology, and your ideas on any interesting subject.

Friends; thank you for always sharing cool ideas, that expand my horizon, and for many good times.

Parents Lena and Martin; thank you for unconditional love, and for letting me be curious, explore and find my own path. Parents-in-law Agneta and Billy; thank you for always making me feel welcome and included.

Sisters Sandra and Ylva; thank you for always being forthright and courageous.

My wife Karin; thank you for your optimism and energy, and for embarking on a great journey with me. My children Tintin and Otto; thank you for letting me see the world anew.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65(1):5-29.
2. Engholm G, Ferlay J, Christensen N, Kejs AMT, Johannesen TB, Khan S, et al. NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 7.1 (09.07.2015). : Association of the Nordic Cancer Registries. Danish Cancer Society.; 2015. Available from: http://www.ancr.nu.
3. Carpelan-Holmstrom M, Nordling S, Pukkala E, Sankila R, Luttges J, Kloppel G, et al. Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry. Gut. 2005;54(3):385-7.
4. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 2014;74(11):2913-21.
5. Klint A, Engholm G, Storm HH, Tryggvadottir L, Gislum M, Hakulinen T, et al. Trends in survival of patients diagnosed with cancer of the digestive organs in the Nordic countries 1964-2003 followed up to the end of 2006. Acta Oncol. 2010;49(5):578-607.
6. Faivre J, Trama A, De Angelis R, Elferink M, Siesling S, Audisio R, et al. Incidence, prevalence and survival of patients with rare epithelial digestive cancers diagnosed in Europe in 1995-2002. Eur J Cancer. 2012;48(10):1417-24.
7. Iodice S, Gandini S, Maisonneuve P, Lowenfels AB. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. Langenbecks Arch Surg. 2008;393(4):535-45.
8. Hassan MM, Bondy ML, Wolff RA, Abbruzzese JL, Vauthey JN, Pisters PW, et al. Risk factors for pancreatic cancer: case-control study. Am J Gastroenterol. 2007;102(12):2696-707.
9. Luo J, Ye W, Zendehdel K, Adami J, Adami HO, Boffetta P, et al. Oral use of Swedish moist snuff (snus) and risk for cancer of the mouth, lung, and pancreas in male construction workers: a retrospective cohort study. Lancet. 2007;369(9578):2015-20.
10. Roosaar A, Johansson AL, Sandborgh-Englund G, Axell T, Nyren O. Cancer and mortality among users and nonusers of snus. Int J Cancer. 2008;123(1):168-73.
11. Boffetta P, Aagnes B, Weiderpass E, Andersen A. Smokeless tobacco use and risk of cancer of the pancreas and other organs. Int J Cancer. 2005;114(6):992-5.
12. Huxley R, Ansary-Moghaddam A, Berrington de Gonzalez A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. Br J Cancer. 2005;92(11):2076-83.

13. Hsu PC, Lin WH, Kuo TH, Lee HM, Kuo C, Li CY. A Population-Based Cohort Study of All-Cause and Site-Specific Cancer Incidence Among Patients With Type 1 Diabetes Mellitus in Taiwan. J Epidemiol. 2015;25(9):567-73.
14. Zendehdel K, Nyren O, Ostenson CG, Adami HO, Ekbom A, Ye W. Cancer incidence in patients with type 1 diabetes mellitus: a population-based cohort study in Sweden. J Natl Cancer Inst. 2003;95(23):1797-800.
15. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med. 2003;348(17):1625-38.
16. Michaud DS, Skinner HG, Wu K, Hu F, Giovannucci E, Willett WC, et al. Dietary patterns and pancreatic cancer risk in men and women. J Natl Cancer Inst. 2005;97(7):518-24.
17. van Kaick G, Wesch H, Luhrs H, Liebermann D, Kaul A. Neoplastic diseases induced by chronic alpha-irradiation--epidemiological, biophysical and clinical results of the German Thorotrast Study. J Radiat Res. 1991;32 Suppl 2:20-33.
18. Coats M, Shimi SM. Cholecystectomy and the risk of alimentary tract cancers: a systematic review. World J Gastroenterol. 2015;21(12):3679-93.
19. Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout M, et al. Liver fluke induces cholangiocarcinoma. PLoS Med. 2007;4(7):e201.
20. Ghadirian P, Boyle P, Simard A, Baillargeon J, Maisonneuve P, Perret C. Reported family aggregation of pancreatic cancer within a population-based case-control study in the Francophone community in Montreal, Canada. Int J Pancreatol. 1991;10(3-4):183-96.
21. Klein AP, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, et al. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. Cancer Res. 2004;64(7):2634-8.
22. Rustgi AK. Familial pancreatic cancer: genetic advances. Genes Dev. 2014;28(1):1-7.
23. Roberts NJ, Norris AL, Petersen GM, Bondy ML, Brand R, Gallinger S, et al. Whole Genome Sequencing Defines the Genetic Heterogeneity of Familial Pancreatic Cancer. Cancer Discov. 2016;6(2):166-75.
24. Couch FJ, Johnson MR, Rabe KG, Brune K, de Andrade M, Goggins M, et al. The prevalence of BRCA2 mutations in familial pancreatic cancer. Cancer Epidemiol Biomarkers Prev. 2007;16(2):342-6.
25. Hahn SA, Greenhalf B, Ellis I, Sina-Frey M, Rieder H, Korte B, et al. BRCA2 germline mutations in familial pancreatic carcinoma. J Natl Cancer Inst. 2003;95(3):214-21.
26. Murphy KM, Brune KA, Griffin C, Sollenberger JE, Petersen GM, Bansal R, et al. Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: deleterious BRCA2 mutations in 17%. Cancer Res. 2002;62(13):3789-93.
27. Breast Cancer Linkage C. Cancer risks in BRCA2 mutation carriers. J Natl Cancer Inst. 1999;91(15):1310-6.
28. van Asperen CJ, Brohet RM, Meijers-Heijboer EJ, Hoogerbrugge N, Verhoef S, Vasen HF, et al. Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. J Med Genet. 2005;42(9):711-9.

29. Liede A, Karlan BY, Narod SA. Cancer risks for male carriers of germline mutations in BRCA1 or BRCA2: a review of the literature. J Clin Oncol. 2004;22(4):735-42.
30. Thompson D, Easton DF, Breast Cancer Linkage C. Cancer Incidence in BRCA1 mutation carriers. J Natl Cancer Inst. 2002;94(18):1358-65.
31. Laitman Y, Keinan Boker L, Liphsitz I, Weissglas-Volkov D, Litz-Philipsborn S, Schayek H, et al. Cancer risks in Jewish male BRCA1 and BRCA2 mutation carriers. Breast Cancer Res Treat. 2015;150(3):631-5.
32. Holter S, Borgida A, Dodd A, Grant R, Semotiuk K, Hedley D, et al. Germline BRCA Mutations in a Large Clinic-Based Cohort of Patients With Pancreatic Adenocarcinoma. J Clin Oncol. 2015;33(28):3124-9.
33. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res. 2015;43(Database issue):D805-11.
34. Goggins M, Hruban RH, Kern SE. BRCA2 is inactivated late in the development of pancreatic intraepithelial neoplasia: evidence and implications. Am J Pathol. 2000;156(5):1767-71.
35. Jones S, Hruban RH, Kamiyama M, Borges M, Zhang X, Parsons DW, et al. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. Science. 2009;324(5924):217.
36. Slater EP, Langer P, Niemczyk E, Strauch K, Butler J, Habbe N, et al. PALB2 mutations in European familial pancreatic cancer families. Clin Genet. 2010;78(5):490-4.
37. Roberts NJ, Jiao Y, Yu J, Kopelovich L, Petersen GM, Bondy ML, et al. ATM mutations in patients with hereditary pancreatic cancer. Cancer Discov. 2012;2(1):41-6.
38. Goldstein AM, Fraser MC, Struewing JP, Hussussian CJ, Ranade K, Zametkin DP, et al. Increased risk of pancreatic cancer in melanoma-prone kindreds with p16INK4 mutations. N Engl J Med. 1995;333(15):970-4.
39. Korsse SE, Harinck F, van Lier MG, Biermann K, Offerhaus GJ, Krak N, et al. Pancreatic cancer risk in Peutz-Jeghers syndrome patients: a large cohort study and implications for surveillance. J Med Genet. 2013;50(1):59-64.
40. Bjork J, Akerbrant H, Iselius L, Bergman A, Engwall Y, Wahlstrom J, et al. Periampullary adenomas and adenocarcinomas in familial adenomatous polyposis: cumulative risks and APC gene mutations. Gastroenterology. 2001;121(5):1127-35.
41. Elkharwily A, Gottlieb K. The pancreas in familial adenomatous polyposis. JOP. 2008;9(1):9-18.
42. Win AK, Lindor NM, Young JP, Macrae FA, Young GP, Williamson E, et al. Risks of primary extracolonic cancers following colorectal cancer in lynch syndrome. J Natl Cancer Inst. 2012;104(18):1363-72.
43. Vasen HF, Wijnen JT, Menko FH, Kleibeuker JH, Taal BG, Griffioen G, et al. Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. Gastroenterology. 1996;110(4):1020-7.
44. Kastrinos F, Mukherjee B, Tayob N, Wang F, Sparr J, Raymond VM, et al. Risk of pancreatic cancer in families with Lynch syndrome. JAMA. 2009;302(16):1790-5.

45. Overman MJ, Pozadzides J, Kopetz S, Wen S, Abbruzzese JL, Wolff RA, et al. Immunophenotype and molecular characterisation of adenocarcinoma of the small intestine. Br J Cancer. 2010;102(1):144-50.
46. Planck M, Ericson K, Piotrowska Z, Halvarsson B, Rambech E, Nilbert M. Microsatellite instability and expression of MLH1 and MSH2 in carcinomas of the small intestine. Cancer. 2003;97(6):1551-7.
47. Aparicio T, Svrcek M, Zaanan A, Beohou E, Laforest A, Afchain P, et al. Small bowel adenocarcinoma phenotyping, a clinicobiological prognostic study. Br J Cancer. 2013;109(12):3057-66.
48. Solomon T RB, Whitehead S, Coleman MP. Cancer Survival in England: Patients Diagnosed 2007–2011 and Followed up to 2012. Office for National Statistics2013.
49. Bilimoria KY, Bentrem DJ, Ko CY, Ritchey J, Stewart AK, Winchester DP, et al. Validation of the 6th edition AJCC Pancreatic Cancer Staging System: report from the National Cancer Database. Cancer. 2007;110(4):738-44.
50. Gillen S, Schuster T, Meyer Zum Buschenfelde C, Friess H, Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med. 2010;7(4):e1000267.
51. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med. 2011;364(19):1817-25.
52. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013;369(18):1691-703.
53. Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997;15(6):2403-13.
54. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. JAMA. 2010;304(10):1073-81.
55. Yeo CJ, Cameron JL, Sohn TA, Lillemoe KD, Pitt HA, Talamini MA, et al. Six hundred fifty consecutive pancreaticoduodenectomies in the 1990s: pathology, complications, and outcomes. Ann Surg. 1997;226(3):248-57; discussion 57-60.
56. Howe JR, Klimstra DS, Moccia RD, Conlon KC, Brennan MF. Factors predictive of survival in ampullary carcinoma. Ann Surg. 1998;228(1):87-94.
57. Qiao QL, Zhao YG, Ye ML, Yang YM, Zhao JX, Huang YT, et al. Carcinoma of the ampulla of Vater: factors influencing long-term survival of 127 patients with resection. World J Surg. 2007;31(1):137-43; discussion 44-6.
58. Jang JY, Kim SW, Park DJ, Ahn YJ, Yoon YS, Choi MG, et al. Actual long-term outcome of extrahepatic bile duct cancer after surgical resection. Ann Surg. 2005;241(1):77-84.
59. Bakaeen FG, Murr MM, Sarr MG, Thompson GB, Farnell MB, Nagorney DM, et al. What prognostic factors are important in duodenal adenocarcinoma? Arch Surg. 2000;135(6):635-41; discussion 41-2.

60. Tsuchiya R, Noda T, Harada N, Miyamoto T, Tomioka T, Yamamoto K, et al. Collective review of small carcinomas of the pancreas. Ann Surg. 1986;203(1):77-81.
61. Ishikawa O, Ohigashi H, Imaoka S, Nakaizumi A, Uehara H, Kitamura T, et al. Minute carcinoma of the pancreas measuring 1 cm or less in diameter--collective review of Japanese case reports. Hepatogastroenterology. 1999;46(25):8-15.
62. Pannala R, Basu A, Petersen GM, Chari ST. New-onset diabetes: a potential clue to the early diagnosis of pancreatic cancer. Lancet Oncol. 2009;10(1):88-95.
63. Furuya N, Kawa S, Akamatsu T, Furihata K. Long-term follow-up of patients with chronic pancreatitis and K-ras gene mutation detected in pancreatic juice. Gastroenterology. 1997;113(2):593-8.
64. Tada M, Ohashi M, Shiratori Y, Okudaira T, Komatsu Y, Kawabe T, et al. Analysis of K-ras gene mutation in hyperplastic duct cells of the pancreas without pancreatic disease. Gastroenterology. 1996;110(1):227-31.
65. Javeed N, Sagar G, Dutta SK, Smyrk TC, Lau JS, Bhattacharya S, et al. Pancreatic Cancer-Derived Exosomes Cause Paraneoplastic beta-cell Dysfunction. Clin Cancer Res. 2015;21(7):1722-33.
66. Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. Eur J Surg Oncol. 2007;33(3):266-70.
67. Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, Anderson RN, et al. Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. Cancer. 2010;116(3):544-73.
68. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010;467(7319):1114-7.
69. Karlson BM, Ekbom A, Lindgren PG, Kallskog V, Rastad J. Abdominal US for diagnosis of pancreatic tumor: prospective cohort analysis. Radiology. 1999;213(1):107-11.
70. Smith SL, Rajan PS. Imaging of pancreatic adenocarcinoma with emphasis on multidetector CT. Clin Radiol. 2004;59(1):26-38.
71. Varghese JC, Farrell MA, Courtney G, Osborne H, Murray FE, Lee MJ. Role of MR cholangiopancreatography in patients with failed or inadequate ERCP. AJR Am J Roentgenol. 1999;173(6):1527-33.
72. Are C, Dhir M, Ravipati L. History of pancreaticoduodenectomy: early misconceptions, initial milestones and the pioneers. HPB (Oxford). 2011;13(6):377-84.
73. Morris PJ, Nardi GL. Pancreaticoduodenal cancer. Experience from 1951 to 1960 with a look ahead and behind. Arch Surg. 1966;92(6):834-7.
74. Gilsdorf RB, Spanos P. Factors influencing morbidity and mortality in pancreaticoduodenectomy. Ann Surg. 1973;177(3):332-7.
75. Crist DW, Sitzmann JV, Cameron JL. Improved hospital morbidity, mortality, and survival after the Whipple procedure. Ann Surg. 1987;206(3):358-65.

76. Trede M, Schwall G, Saeger HD. Survival after pancreatoduodenectomy. 118 consecutive resections without an operative mortality. Ann Surg. 1990;211(4):447-58.
77. Bottger TC, Junginger T. Factors influencing morbidity and mortality after pancreaticoduodenectomy: critical analysis of 221 resections. World J Surg. 1999;23(2):164-71; discussion 71-2.
78. Cameron JL, Riall TS, Coleman J, Belcher KA. One thousand consecutive pancreaticoduodenectomies. Ann Surg. 2006;244(1):10-5.
79. Fernandez-del Castillo C, Rattner DW, Warshaw AL. Standards for pancreatic resection in the 1990s. Arch Surg. 1995;130(3):295-9; discussion 9-300.
80. Herrera-Cabezon FJ, Sanchez-Acedo P, Zazpe-Ripa C, Tarifa-Castilla A, Lera-Tricas JM. Quality standards in 480 pancreatic resections: a prospective observational study. Rev Esp Enferm Dig. 2015;107(3):143-51.
81. Gouma DJ, van Geenen RC, van Gulik TM, de Haan RJ, de Wit LT, Busch OR, et al. Rates of complications and death after pancreaticoduodenectomy: risk factors and the impact of hospital volume. Ann Surg. 2000;232(6):786-95.
82. Birkmeyer JD, Stukel TA, Siewers AE, Goodney PP, Wennberg DE, Lucas FL. Surgeon volume and operative mortality in the United States. N Engl J Med. 2003;349(22):2117-27.
83. Geer RJ, Brennan MF. Prognostic indicators for survival after resection of pancreatic adenocarcinoma. Am J Surg. 1993;165(1):68-72; discussion -3.
84. Spadi R, Brusa F, Ponzetti A, Chiappino I, Birocco N, Ciuffreda L, et al. Current therapeutic strategies for advanced pancreatic cancer: A review for clinicians. World J Clin Oncol. 2016;7(1):27-43.
85. Palmer KR, Kerr M, Knowles G, Cull A, Carter DC, Leonard RC. Chemotherapy prolongs survival in inoperable pancreatic carcinoma. Br J Surg. 1994;81(6):882-5.
86. Bakkevold KE, Arnesjo B, Dahl O, Kambestad B. Adjuvant combination chemotherapy (AMF) following radical resection of carcinoma of the pancreas and papilla of Vater--results of a controlled, prospective, randomised multicentre study. Eur J Cancer. 1993;29A(5):698-703.
87. Regine WF, Winter KA, Abrams RA, Safran H, Hoffman JP, Konski A, et al. Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial. JAMA. 2008;299(9):1019-26.
88. Sudo K, Nakamura K, Yamaguchi T. S-1 in the treatment of pancreatic cancer. World J Gastroenterol. 2014;20(41):15110-8.
89. Neoptolemos JP, Moore MJ, Cox TF, Valle JW, Palmer DH, McDonald AC, et al. Effect of adjuvant chemotherapy with fluorouracil plus folinic acid or gemcitabine vs observation on survival in patients with resected periampullary adenocarcinoma: the ESPAC-3 periampullary cancer randomized trial. JAMA. 2012;308(2):147-56.
90. Overman MJ. Recent advances in the management of adenocarcinoma of the small intestine. Gastrointest Cancer Res. 2009;3(3):90-6.
91. Yachida S, Wood LD, Suzuki M, Takai E, Totoki Y, Kato M, et al. Genomic Sequencing Identifies ELF3 as a Driver of Ampullary Carcinoma. Cancer Cell. 2016.

92. Westgaard A, Pomianowska E, Clausen OP, Gladhaug IP. Intestinal-type and pancreatobiliary-type adenocarcinomas: how does ampullary carcinoma differ from other periampullary malignancies? Ann Surg Oncol. 2013;20(2):430-9.
93. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med. 2010;362(14):1273-81.
94. ESPAC-4. European Study Group for Pancreatic Cancer - Trial 4. Combination versus single agent chemotherapy in resectable pancreatic ductal and peri-ampullary cancers. UK Clinical Research Network: Cancer Research UK; Available from: http://public.ukcrn.org.uk/Search/StudyDetail.aspx?StudyID=4307.
95. Mallinson CN, Rake MO, Cocking JB, Fox CA, Cwynarski MT, Diffey BL, et al. Chemotherapy in pancreatic cancer: results of a controlled, prospective, randomised, multicentre trial. Br Med J. 1980;281(6255):1589-91.
96. Schultheis B, Strumberg D, Bergmann L, Graeven U, Hanauske AR, Lipp R, et al. Results of a phase II trial of S-1 as first-line treatment of metastatic pancreatic cancer (CESAR-study group). Invest New Drugs. 2012;30(3):1184-92.
97. Di Marco M, Di Cicilia R, Macchini M, Nobili E, Vecchiarelli S, Brandi G, et al. Metastatic pancreatic cancer: is gemcitabine still the best standard treatment? (Review). Oncol Rep. 2010;23(5):1183-92.
98. Cunningham D, Chau I, Stocken DD, Valle JW, Smith D, Steward W, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol. 2009;27(33):5513-8.
99. Sultana A, Smith CT, Cunningham D, Starling N, Neoptolemos JP, Ghaneh P. Meta-analyses of chemotherapy for locally advanced and metastatic pancreatic cancer. J Clin Oncol. 2007;25(18):2607-15.
100. Conroy T, Paillot B, Francois E, Bugat R, Jacob JH, Stein U, et al. Irinotecan plus oxaliplatin and leucovorin-modulated fluorouracil in advanced pancreatic cancer--a Groupe Tumeurs Digestives of the Federation Nationale des Centres de Lutte Contre le Cancer study. J Clin Oncol. 2005;23(6):1228-36.
101. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol. 2007;25(15):1960-6.
102. Di Marco M, Grassi E, Durante S, Vecchiarelli S, Palloni A, Macchini M, et al. State of the art biological therapies in pancreatic cancer. World J Gastrointest Oncol. 2016;8(1):55-66.
103. Rutter CE, Park HS, Corso CD, Lester-Coll NH, Mancini BR, Yeboa DN, et al. Addition of radiotherapy to adjuvant chemotherapy is associated with improved overall survival in resected pancreatic adenocarcinoma: An analysis of the National Cancer Data Base. Cancer. 2015;121(23):4141-9.
104. Liao WC, Chien KL, Lin YL, Wu MS, Lin JT, Wang HP, et al. Adjuvant treatments for resected pancreatic adenocarcinoma: a systematic review and network meta-analysis. Lancet Oncol. 2013;14(11):1095-103.
105. Hazard L. The role of radiation therapy in pancreas cancer. Gastrointest Cancer Res. 2009;3(1):20-8.

106. Hammel P, Huguet F, Van Laethem JL, Goldstein D, Glimelius B, Artru P, et al. Comparison of chemoradiotherapy (CRT) and chemotherapy (CT) in patients with a locally advanced pancreatic cancer (LAPC) controlled after 4 months of gemcitabine with or without erlotinib: Final results of the international phase III LAP 07 study. 2013 ASCO Annual Meeting: J Clin Oncol 31, 2013 (suppl; abstr LBA4003); 2013.
107. Habermehl D, Brecht IC, Debus J, Combs SE. Palliative radiation therapy in patients with metastasized pancreatic cancer - description of a rare patient group. Eur J Med Res. 2014;19:24.
108. Heestand GM, Kurzrock R. Molecular landscape of pancreatic cancer: implications for current clinical trials. Oncotarget. 2015.
109. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature. 2010;467(7319):1109-13.
110. Waddell N, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. Nature. 2015;518(7540):495-501.
111. Lowery MA, Kelsen DP, Stadler ZK, Yu KH, Janjigian YY, Ludwig E, et al. An emerging entity: pancreatic adenocarcinoma associated with a known BRCA mutation: clinical descriptors, treatment implications, and future directions. Oncologist. 2011;16(10):1397-402.
112. Golan T, Kanji ZS, Epelbaum R, Devaud N, Dagan E, Holter S, et al. Overall survival and clinical characteristics of pancreatic cancer in BRCA mutation carriers. Br J Cancer. 2014;111(6):1132-8.
113. Sharma MB, Carus A, Sunde L, Hamilton-Dutoit S, Ladekarl M. BRCA-associated pancreatico-biliary neoplasms: Four cases illustrating the emerging clinical impact of genotyping. Acta Oncol. 2016;55(3):377-81.
114. Sandhu SK, Yap TA, de Bono JS. Poly(ADP-ribose) polymerase inhibitors in cancer treatment: a clinical perspective. Eur J Cancer. 2010;46(1):9-20.
115. Livraghi L, Garber JE. PARP inhibitors in the management of breast cancer: current data and future prospects. BMC Med. 2015;13:188.
116. Kaufman B, Shapira-Frommer R, Schmutzler RK, Audeh MW, Friedlander M, Balmana J, et al. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. J Clin Oncol. 2015;33(3):244-50.
117. Chmielecki J, Ross JS, Wang K, Frampton GM, Palmer GA, Ali SM, et al. Oncogenic alterations in ERBB2/HER2 represent potential therapeutic targets across tumors from diverse anatomic sites of origin. Oncologist. 2015;20(1):7-12.
118. Samartzis EP, Gutsche K, Dedes KJ, Fink D, Stucki M, Imesch P. Loss of ARID1A expression sensitizes cancer cells to PI3K- and AKT-inhibition. Oncotarget. 2014;5(14):5295-303.
119. Domingo E, Church DN, Sieber O, Ramamoorthy R, Yanagisawa Y, Johnstone E, et al. Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. J Clin Oncol. 2013;31(34):4297-305.
120. Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature. 2012;483(7387):100-3.

121. Van Cutsem E, Atreya C, André T, Bendell J, Schellens J, Gordon M, et al. Updated Results of the MEK inhibitor trametinib (T), BRAF inhibitor dabrafenib (D), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E mutated (BRAFm) metastatic colorectal cancer (mCRC) Ann Oncol. 2015;26(suppl 4):iv119.
122. Hirai H, Iwasawa Y, Okada M, Arai T, Nishibata T, Kobayashi M, et al. Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents. Mol Cancer Ther. 2009;8(11):2992-3000.
123. Toledo F, Wahl GM. MDM2 and MDM4: p53 regulators as targets in anticancer therapy. Int J Biochem Cell Biol. 2007;39(7-8):1476-82.
124. Zhao Y, Aguilar A, Bernard D, Wang S. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction (MDM2 Inhibitors) in clinical trials for cancer treatment. J Med Chem. 2015;58(3):1038-52.
125. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. Nucleic Acids Res. 1998;26(15):3453-9.
126. Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer. 2006;6(12):909-23.
127. Forslund A, Zeng Z, Qin LX, Rosenberg S, Ndubuisi M, Pincas H, et al. MDM2 gene amplification is correlated to tumor progression but not to the presence of SNP309 or TP53 mutational status in primary colorectal cancers. Mol Cancer Res. 2008;6(2):205-11.
128. Gunther T, Schneider-Stock R, Hackel C, Kasper HU, Pross M, Hackelsberger A, et al. Mdm2 gene amplification in gastric cancer correlation with expression of Mdm2 protein and p53 alterations. Mod Pathol. 2000;13(6):621-6.
129. Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. Clin Cancer Res. 2000;6(8):2969-72.
130. Blackford A, Parmigiani G, Kensler TW, Wolfgang C, Jones S, Zhang X, et al. Genetic mutations associated with cigarette smoking in pancreatic cancer. Cancer Res. 2009;69(8):3681-8.
131. Pellegata NS, Sessa F, Renault B, Bonato M, Leone BE, Solcia E, et al. K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. Cancer Res. 1994;54(6):1556-60.
132. Hruban RH, van Mansfeld AD, Offerhaus GJ, van Weering DH, Allison DC, Goodman SN, et al. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. Am J Pathol. 1993;143(2):545-54.
133. Smit VT, Boot AJ, Smits AM, Fleuren GJ, Cornelisse CJ, Bos JL. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. Nucleic Acids Res. 1988;16(16):7773-82.
134. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature. 2012;491(7424):399-405.
135. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. Lancet. 2016.
136. Wood LD, Hruban RH. Pathology and molecular genetics of pancreatic neoplasms. Cancer J. 2012;18(6):492-501.

137. McCarthy DM, Hruban RH, Argani P, Howe JR, Conlon KC, Brennan MF, et al. Role of the DPC4 tumor suppressor gene in adenocarcinoma of the ampulla of Vater: analysis of 140 cases. Mod Pathol. 2003;16(3):272-8.
138. Laforest A, Aparicio T, Zaanan A, Silva FP, Didelot A, Desbeaux A, et al. ERBB2 gene as a potential therapeutic target in small bowel adenocarcinoma. Eur J Cancer. 2014;50(10):1740-6.
139. Spaargaren M, Bischoff JR, McCormick F. Signal transduction by Ras-like GTPases: a potential target for anticancer drugs. Gene Expr. 1995;4(6):345-56.
140. di Magliano MP, Logsdon CD. Roles for KRAS in pancreatic tumor development and progression. Gastroenterology. 2013;144(6):1220-9.
141. Boeck S, Jung A, Laubender RP, Neumann J, Egg R, Goritschan C, et al. KRAS mutation status is not predictive for objective response to anti-EGFR treatment with erlotinib in patients with advanced pancreatic cancer. J Gastroenterol. 2013;48(4):544-8.
142. Huang J, Lohr JM, Nilsson M, Segersvard R, Matsson H, Verbeke C, et al. Variant Profiling of Candidate Genes in Pancreatic Ductal Adenocarcinoma. Clin Chem. 2015;61(11):1408-16.
143. Weissmueller S, Manchado E, Saborowski M, Morris JPt, Wagenblast E, Davis CA, et al. Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling. Cell. 2014;157(2):382-94.
144. Wade M, Li YC, Wahl GM. MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nat Rev Cancer. 2013;13(2):83-96.
145. Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. Nat Rev Cancer. 2001;1(3):233-40.
146. Masciarelli S, Fontemaggi G, Di Agostino S, Donzelli S, Carcarino E, Strano S, et al. Gain-of-function mutant p53 downregulates miR-223 contributing to chemoresistance of cultured tumor cells. Oncogene. 2014;33(12):1601-8.
147. Schutte M, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK, et al. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res. 1997;57(15):3126-30.
148. Caldas C, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, et al. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nat Genet. 1994;8(1):27-32.
149. Attri J, Srinivasan R, Majumdar S, Radotra BD, Wig J. Alterations of tumor suppressor gene p16INK4a in pancreatic ductal carcinoma. BMC Gastroenterol. 2005;5:22.
150. Chang DL, Qiu W, Ying H, Zhang Y, Chen CY, Xiao ZX. ARF promotes accumulation of retinoblastoma protein through inhibition of MDM2. Oncogene. 2007;26(32):4627-34.
151. Shima K, Nosho K, Baba Y, Cantor M, Meyerhardt JA, Giovannucci EL, et al. Prognostic significance of CDKN2A (p16) promoter methylation and loss of expression in 902 colorectal cancers: Cohort study and literature review. Int J Cancer. 2011;128(5):1080-94.
152. Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, Weinstein CL, et al. DPC4 gene in various tumor types. Cancer Res. 1996;56(11):2527-30.

153. Wilentz RE, Su GH, Dai JL, Sparks AB, Argani P, Sohn TA, et al. Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas : a new marker of DPC4 inactivation. Am J Pathol. 2000;156(1):37-43.
154. Argani P, Shaukat A, Kaushal M, Wilentz RE, Su GH, Sohn TA, et al. Differing rates of loss of DPC4 expression and of p53 overexpression among carcinomas of the proximal and distal bile ducts. Cancer. 2001;91(7):1332-41.
155. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor-beta signaling in cancer. J Natl Cancer Inst. 2000;92(17):1388-402.
156. Hruban RH, Offerhaus GJ, Kern SE, Goggins M, Wilentz RE, Yeo CJ. Tumor-suppressor genes in pancreatic cancer. J Hepatobiliary Pancreat Surg. 1998;5(4):383-91.
157. Iacobuzio-Donahue CA, Fu B, Yachida S, Luo M, Abe H, Henderson CM, et al. DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. J Clin Oncol. 2009;27(11):1806-13.
158. Oshima M, Okano K, Muraki S, Haba R, Maeba T, Suzuki Y, et al. Immunohistochemically detected expression of 3 major genes (CDKN2A/p16, TP53, and SMAD4/DPC4) strongly predicts survival in patients with resectable pancreatic cancer. Ann Surg. 2013;258(2):336-46.
159. Yachida S, White CM, Naito Y, Zhong Y, Brosnan JA, Macgregor-Das AM, et al. Clinical significance of the genetic landscape of pancreatic cancer and implications for identification of potential long-term survivors. Clin Cancer Res. 2012;18(22):6339-47.
160. Nakamura H, Arai Y, Totoki Y, Shirota T, Elzawahry A, Kato M, et al. Genomic spectra of biliary tract cancer. Nat Genet. 2015;47(9):1003-10.
161. Guan B, Wang TL, Shih Ie M. ARID1A, a factor that promotes formation of SWI/SNF-mediated chromatin remodeling, is a tumor suppressor in gynecologic cancers. Cancer Res. 2011;71(21):6718-27.
162. Garcia-Murillas I, Sharpe R, Pearson A, Campbell J, Natrajan R, Ashworth A, et al. An siRNA screen identifies the GNAS locus as a driver in 20q amplified breast cancer. Oncogene. 2014;33(19):2478-86.
163. Wu J, Matthaei H, Maitra A, Dal Molin M, Wood LD, Eshleman JR, et al. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. Sci Transl Med. 2011;3(92):92ra66.
164. Tumours of the ampullary region. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, editors. World Health Organization Classification of Tumours of Tumours of the Digestive System. 4th ed. Lyon: IARC Press; 2010. p. 87-92.
165. Hruban RH, Bishop Pitman M, Klimstra DS. Tumors of the pancreas. In Atlas of Tumor Pathology. Washington: Armed Forces Institute of Pathology; 2007.
166. Bosman FT, Carneiro F, Hruban RH, Theise ND. Tumours of the ampullary region. WHO Classification of Tumours of the Digestive System. 4 ed. Lyon: International Agency for Research on Cancer; 2010. p. 87-91.
167. Pomianowska E, Grzyb K, Westgaard A, Clausen OP, Gladhaug IP. Reclassification of tumour origin in resected periampullary adenocarcinomas reveals underestimation of distal bile duct cancer. Eur J Surg Oncol. 2012;38(11):1043-50.

168. Verbeke CS, Gladhaug IP. Resection margin involvement and tumour origin in pancreatic head cancer. Br J Surg. 2012;99(8):1036-49.
169. Westgaard A, Clausen OP, Gladhaug IP. Survival estimates after pancreatoduodenectomy skewed by non-standardized histopathology reports. APMIS. 2011;119(10):689-700.
170. Jung KW, Park S, Kong HJ, Won YJ, Boo YK, Shin HR, et al. Cancer statistics in Korea: incidence, mortality and survival in 2006-2007. J Korean Med Sci. 2010;25(8):1113-21.
171. Kim RD, Kundhal PS, McGilvray ID, Cattral MS, Taylor B, Langer B, et al. Predictors of failure after pancreaticoduodenectomy for ampullary carcinoma. J Am Coll Surg. 2006;202(1):112-9.
172. Michelassi F, Erroi F, Dawson PJ, Pietrabissa A, Noda S, Handcock M, et al. Experience with 647 consecutive tumors of the duodenum, ampulla, head of the pancreas, and distal common bile duct. Ann Surg. 1989;210(4):544-54; discussion 54-6.
173. Bilimoria KY, Bentrem DJ, Wayne JD, Ko CY, Bennett CL, Talamonti MS. Small bowel cancer in the United States: changes in epidemiology, treatment, and survival over the last 20 years. Ann Surg. 2009;249(1):63-71.
174. Overman MJ, Zhang J, Kopetz S, Davies M, Jiang ZQ, Stemke-Hale K, et al. Gene expression profiling of ampullary carcinomas classifies ampullary carcinomas into biliary-like and intestinal-like subtypes that are prognostic of outcome. PLoS One. 2013;8(6):e65144.
175. Outerbridge GW. VII. Carcinoma of the Papilla of Vater. Ann Surg. 1913;57(3):402-26.
176. Kimura W, Futakawa N, Yamagata S, Wada Y, Kuroda A, Muto T, et al. Different clinicopathologic findings in two histologic types of carcinoma of papilla of Vater. Jpn J Cancer Res. 1994;85(2):161-6.
177. Bronsert P, Kohler I, Werner M, Makowiec F, Kuesters S, Hoeppner J, et al. Intestinal-type of differentiation predicts favourable overall survival: confirmatory clinicopathological analysis of 198 periampullary adenocarcinomas of pancreatic, biliary, ampullary and duodenal origin. BMC Cancer. 2013;13:428.
178. Kumari N, Prabha K, Singh RK, Baitha DK, Krishnani N. Intestinal and pancreatobiliary differentiation in periampullary carcinoma: the role of immunohistochemistry. Hum Pathol. 2013;44(10):2213-9.
179. Carter JT, Grenert JP, Rubenstein L, Stewart L, Way LW. Tumors of the ampulla of vater: histopathologic classification and predictors of survival. J Am Coll Surg. 2008;207(2):210-8.
180. de Paiva Haddad LB, Patzina RA, Penteado S, Montagnini AL, da Cunha JE, Machado MC, et al. Lymph node involvement and not the histophatologic subtype is correlated with outcome after resection of adenocarcinoma of the ampulla of vater. J Gastrointest Surg. 2010;14(4):719-28.
181. Lowe MC, Coban I, Adsay NV, Sarmiento JM, Chu CK, Staley CA, et al. Important prognostic factors in adenocarcinoma of the ampulla of Vater. Am Surg. 2009;75(9):754-60; discussion 61.

182. Westgaard A, Tafjord S, Farstad IN, Cvancarova M, Eide TJ, Mathisen O, et al. Pancreatobiliary versus intestinal histologic type of differentiation is an independent prognostic factor in resected periampullary adenocarcinoma. BMC Cancer. 2008;8:170.
183. Ang DC, Shia J, Tang LH, Katabi N, Klimstra DS. The utility of immunohistochemistry in subtyping adenocarcinoma of the ampulla of vater. Am J Surg Pathol. 2014;38(10):1371-9.
184. Romiti A, Barucca V, Zullo A, Sarcina I, Di Rocco R, D'Antonio C, et al. Tumors of ampulla of Vater: A case series and review of chemotherapy options. World J Gastrointest Oncol. 2012;4(3):60-7.
185. Matsubayashi H, Watanabe H, Yamaguchi T, Ajioka Y, Nishikura K, Kijima H, et al. Differences in mucus and K-ras mutation in relation to phenotypes of tumors of the papilla of vater. Cancer. 1999;86(4):596-607.
186. Perrone G, Morini S, Santini D, Rabitti C, Vincenzi B, Alloni R, et al. Human equilibrative nucleoside transporter 1 and carcinoma of the ampulla of Vater: expression differences in tumour histotypes. Eur J Histochem. 2010;54(3):e38.
187. Elebro J, Jirstrom K. Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma. Diagn Pathol. 2014;9(1):80.
188. Baumhoer D, Zlobec I, Tornillo L, Dietmaier W, Wuensch PH, Hartmann A, et al. Immunophenotyping and oncogene amplifications in tumors of the papilla of Vater. Virchows Arch. 2008;453(6):579-88.
189. Sobin LH, Gospodarowicz MK, Wittekind C. TNM Classification of Malignant Tumours, 7th Edition. 7th ed. Chichester, West Sussex, UK ; Hoboken, NJ: Wiley-Blackwell; 2009. 310 p.
190. Qiao QL, Zhang TP, Guo JC, Zhan HX, Zhao JX, Liu YC, et al. Prognostic factors after pancreatoduodenectomy for distal bile duct cancer. Am Surg. 2011;77(11):1445-8.
191. Bilimoria KY, Talamonti MS, Sener SF, Bilimoria MM, Stewart AK, Winchester DP, et al. Effect of hospital volume on margin status after pancreaticoduodenectomy for cancer. J Am Coll Surg. 2008;207(4):510-9.
192. Hatzaras I, George N, Muscarella P, Melvin WS, Ellison EC, Bloomston M. Predictors of survival in periampullary cancers following pancreaticoduodenectomy. Ann Surg Oncol. 2010;17(4):991-7.
193. Schmidt CM, Powell ES, Yiannoutsos CT, Howard TJ, Wiebke EA, Wiesenauer CA, et al. Pancreaticoduodenectomy: a 20-year experience in 516 patients. Arch Surg. 2004;139(7):718-27.
194. Jarufe NP, Coldham C, Mayer AD, Mirza DF, Buckels JA, Bramhall SR. Favourable prognostic factors in a large UK experience of adenocarcinoma of the head of the pancreas and periampullary region. Dig Surg. 2004;21(3):202-9.
195. Riall TS, Cameron JL, Lillemoe KD, Winter JM, Campbell KA, Hruban RH, et al. Resected periampullary adenocarcinoma: 5-year survivors and their 6- to 10-year follow-up. Surgery. 2006;140(5):764-72.

196. van Geenen RC, van Gulik TM, Offerhaus GJ, de Wit LT, Busch OR, Obertop H, et al. Survival after pancreaticoduodenectomy for periampullary adenocarcinoma: an update. Eur J Surg Oncol. 2001;27(6):549-57.
197. Lim JE, Chien MW, Earle CC. Prognostic factors following curative resection for pancreatic adenocarcinoma: a population-based, linked database analysis of 396 patients. Ann Surg. 2003;237(1):74-85.
198. Yoshida T, Matsumoto T, Sasaki A, Morii Y, Aramaki M, Kitano S. Prognostic factors after pancreatoduodenectomy with extended lymphadenectomy for distal bile duct cancer. Arch Surg. 2002;137(1):69-73.
199. Klein F, Jacob D, Bahra M, Pelzer U, Puhl G, Krannich A, et al. Prognostic factors for long-term survival in patients with ampullary carcinoma: the results of a 15-year observation period after pancreaticoduodenectomy. HPB Surg. 2014;2014:970234.
200. Gutierrez JC, Franceschi D, Koniaris LG. How many lymph nodes properly stage a periampullary malignancy? J Gastrointest Surg. 2008;12(1):77-85.
201. Schwarz RE, Smith DD. Extent of lymph node retrieval and pancreatic cancer survival: information from a large US population database. Ann Surg Oncol. 2006;13(9):1189-200.
202. Pomianowska E, Westgaard A, Mathisen O, Clausen OP, Gladhaug IP. Prognostic relevance of number and ratio of metastatic lymph nodes in resected pancreatic, ampullary, and distal bile duct carcinomas. Ann Surg Oncol. 2013;20(1):233-41.
203. Pawlik TM, Gleisner AL, Cameron JL, Winter JM, Assumpcao L, Lillemoe KD, et al. Prognostic relevance of lymph node ratio following pancreaticoduodenectomy for pancreatic cancer. Surgery. 2007;141(5):610-8.
204. Hruban RH, Fukushima N. Pancreatic adenocarcinoma: update on the surgical pathology of carcinomas of ductal origin and PanINs. Mod Pathol. 2007;20 Suppl 1:S61-70.
205. Yeo CJ, Sohn TA, Cameron JL, Hruban RH, Lillemoe KD, Pitt HA. Periampullary adenocarcinoma: analysis of 5-year survivors. Ann Surg. 1998;227(6):821-31.
206. Wasif N, Ko CY, Farrell J, Wainberg Z, Hines OJ, Reber H, et al. Impact of tumor grade on prognosis in pancreatic cancer: should we include grade in AJCC staging? Ann Surg Oncol. 2010;17(9):2312-20.
207. Garcea G, Dennison AR, Pattenden CJ, Neal CP, Sutton CD, Berry DP. Survival following curative resection for pancreatic ductal adenocarcinoma. A systematic review of the literature. JOP. 2008;9(2):99-132.
208. Sudo T, Murakami Y, Uemura K, Hayashidani Y, Hashimoto Y, Ohge H, et al. Prognostic impact of perineural invasion following pancreatoduodenectomy with lymphadenectomy for ampullary carcinoma. Dig Dis Sci. 2008;53(8):2281-6.
209. Jamieson NB, Foulis AK, Oien KA, Dickson EJ, Imrie CW, Carter R, et al. Peripancreatic fat invasion is an independent predictor of poor outcome following pancreaticoduodenectomy for pancreatic ductal adenocarcinoma. J Gastrointest Surg. 2011;15(3):512-24.
210. Kayahara M, Nagakawa T, Ueno K, Ohta T, Takeda T, Miyazaki I. An evaluation of radical resection for pancreatic cancer based on the mode of recurrence as determined by autopsy and diagnostic imaging. Cancer. 1993;72(7):2118-23.

211. Westerdahl J, Andren-Sandberg A, Ihse I. Recurrence of exocrine pancreatic cancer--local or hepatic? Hepatogastroenterology. 1993;40(4):384-7.
212. Sperti C, Pasquali C, Piccoli A, Pedrazzoli S. Recurrence after resection for ductal adenocarcinoma of the pancreas. World J Surg. 1997;21(2):195-200.
213. Menon KV, Gomez D, Smith AM, Anthoney A, Verbeke CS. Impact of margin status on survival following pancreatoduodenectomy for cancer: the Leeds Pathology Protocol (LEEPP). HPB (Oxford). 2009;11(1):18-24.
214. Verbeke CS, Leitch D, Menon KV, McMahon MJ, Guillou PJ, Anthoney A. Redefining the R1 resection in pancreatic cancer. Br J Surg. 2006;93(10):1232-7.
215. Han HJ, Russo J, Kohwi Y, Kohwi-Shigematsu T. SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. Nature. 2008;452(7184):187-93.
216. Dobreva G, Dambacher J, Grosschedl R. SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression. Genes Dev. 2003;17(24):3048-61.
217. Kohwi-Shigematsu T, Poterlowicz K, Ordinario E, Han H-J, Botchkarev VA, Kohwi Y, et al. Genome organizing function of SATB1 in tumor progression. Semin Cancer Biol. 2013;23(2):72-9.
218. Mao L, Yang C, Wang J, Li W, Wen R, Chen J, et al. SATB1 is overexpressed in metastatic prostate cancer and promotes prostate cancer cell growth and invasion. J Transl Med. 2013;11:111.
219. Zhao XD, Ji WY, Zhang W, He LX, Yang J, Liang HJ, et al. Overexpression of SATB1 in laryngeal squamous cell carcinoma. ORL J Otorhinolaryngol Relat Spec. 2010;72(1):1-5.
220. Shen Z, Zeng Y, Guo J, Wu Y, Jiang X, Ding R, et al. Over-expression of the special AT rich sequence binding protein 1 (SATB1) promotes the progression of nasopharyngeal carcinoma: association with EBV LMP-1 expression. J Transl Med. 2013;11:217.
221. Tu W, Luo M, Wang Z, Yan W, Xia Y, Deng H, et al. Upregulation of SATB1 promotes tumor growth and metastasis in liver cancer. Liver Int. 2012;32(7):1064-78.
222. Meng WJ, Yan H, Zhou B, Zhang W, Kong XH, Wang R, et al. Correlation of SATB1 overexpression with the progression of human rectal cancer. Int J Colorectal Dis. 2012;27(2):143-50.
223. Chen H, Takahara M, Oba J, Xie L, Chiba T, Takeuchi S, et al. Clinicopathologic and prognostic significance of SATB1 in cutaneous malignant melanoma. J Dermatol Sci. 2011;64(1):39-44.
224. Nodin B, Hedner C, Uhlen M, Jirstrom K. Expression of the global regulator SATB1 is an independent factor of poor prognosis in high grade epithelial ovarian cancer. J Ovarian Res. 2012;5(1):24.
225. Chu SH, Ma YB, Feng DF, Zhang H, Zhu ZA, Li ZQ, et al. Upregulation of SATB1 is associated with the development and progression of glioma. J Transl Med. 2012;10:149.
226. Lu X, Cheng C, Zhu S, Yang Y, Zheng L, Wang G, et al. SATB1 is an independent prognostic marker for gastric cancer in a Chinese population. Oncol Rep. 2010;24(4):981-7.

227. Hedner C, Gaber A, Korkocic D, Nodin B, Uhlen M, Kuteeva E, et al. SATB1 is an independent prognostic factor in radically resected upper gastrointestinal tract adenocarcinoma. Virchows Arch. 2014;465(6):649-59.
228. Elebro J, Heby M, Gaber A, Nodin B, Jonsson L, Fristedt R, et al. Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. J Transl Med. 2014;12(1):289.
229. Magnusson K, de Wit M, Brennan DJ, Johnson LB, McGee SF, Lundberg E, et al. SATB2 in combination with cytokeratin 20 identifies over 95% of all colorectal carcinomas. Am J Surg Pathol. 2011;35(7):937-48.
230. Greenhalf W, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, Lamb RF, et al. Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. J Natl Cancer Inst. 2014;106(1):djt347.
231. Marechal R, Bachet JB, Mackey JR, Dalban C, Demetter P, Graham K, et al. Levels of gemcitabine transport and metabolism proteins predict survival times of patients treated with gemcitabine for pancreatic adenocarcinoma. Gastroenterology. 2012;143(3):664-74 e1-6.
232. Wei CH, Gorgan TR, Elashoff DA, Hines OJ, Farrell JJ, Donahue TR. A meta-analysis of gemcitabine biomarkers in patients with pancreaticobiliary cancers. Pancreas. 2013;42(8):1303-10.
233. Santini D, Perrone G, Vincenzi B, Lai R, Cass C, Alloni R, et al. Human equilibrative nucleoside transporter 1 (hENT1) protein is associated with short survival in resected ampullary cancer. Ann Oncol. 2008;19(4):724-8.
234. Bouffard DY, Laliberte J, Momparler RL. Kinetic studies on 2',2'-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem Pharmacol. 1993;45(9):1857-61.
235. Saiki Y, Yoshino Y, Fujimura H, Manabe T, Kudo Y, Shimada M, et al. DCK is frequently inactivated in acquired gemcitabine-resistant human cancer cells. Biochem Biophys Res Commun. 2012;421(1):98-104.
236. McAllister F, Pineda DM, Jimbo M, Lal S, Burkhart RA, Moughan J, et al. dCK expression correlates with 5-fluorouracil efficacy and HuR cytoplasmic expression in pancreatic cancer: a dual-institutional follow-up with the RTOG 9704 trial. Cancer Biol Ther. 2014;15(6):688-98.
237. Elebro J, Ben Dror L, Heby M, Nodin B, Jirstrom K, Eberhard J. Prognostic effect of hENT1, dCK and HuR expression by morphological type in periampullary adenocarcinoma, including pancreatic cancer. Acta Oncol. 2015:1-11.
238. Hinman MN, Lou H. Diverse molecular functions of Hu proteins. Cell Mol Life Sci. 2008;65(20):3168-81.
239. Lopez de Silanes I, Lal A, Gorospe M. HuR: post-transcriptional paths to malignancy. RNA Biol. 2005;2(1):11-3.
240. Kotta-Loizou I, Giaginis C, Theocharis S. Clinical significance of HuR expression in human malignancy. Med Oncol. 2014;31(9):161.
241. Mrena J, Wiksten JP, Thiel A, Kokkola A, Pohjola L, Lundin J, et al. Cyclooxygenase-2 is an independent prognostic factor in gastric cancer and its expression is regulated by the messenger RNA stability factor HuR. Clin Cancer Res. 2005;11(20):7362-8.

242. Sun DP, Lin CY, Tian YF, Chen LT, Lin LC, Lee SW, et al. Clinicopathological significance of HuR expression in gallbladder carcinoma: with special emphasis on the implications of its nuclear and cytoplasmic expression. Tumour Biol. 2013;34(5):3059-69.
243. Heinonen M, Fagerholm R, Aaltonen K, Kilpivaara O, Aittomaki K, Blomqvist C, et al. Prognostic role of HuR in hereditary breast cancer. Clin Cancer Res. 2007;13(23):6959-63.
244. Liang PI, Li WM, Wang YH, Wu TF, Wu WR, Liao AC, et al. HuR cytoplasmic expression is associated with increased cyclin A expression and poor outcome with upper urinary tract urothelial carcinoma. BMC Cancer. 2012;12:611.
245. Wang J, Wang B, Bi J, Zhang C. Cytoplasmic HuR expression correlates with angiogenesis, lymphangiogenesis, and poor outcome in lung cancer. Med Oncol. 2011;28 Suppl 1:S577-85.
246. Costantino CL, Witkiewicz AK, Kuwano Y, Cozzitorto JA, Kennedy EP, Dasgupta A, et al. The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR Up-regulates the expression of the gemcitabine metabolizing enzyme deoxycytidine kinase. Cancer Res. 2009;69(11):4567-72.
247. Richards NG, Rittenhouse DW, Freydin B, Cozzitorto JA, Grenda D, Rui H, et al. HuR status is a powerful marker for prognosis and response to gemcitabine-based chemotherapy for resected pancreatic ductal adenocarcinoma patients. Ann Surg. 2010;252(3):499-505; discussion -6.
248. Yoo PS, Sullivan CA, Kiang S, Gao W, Uchio EM, Chung GG, et al. Tissue microarray analysis of 560 patients with colorectal adenocarcinoma: high expression of HuR predicts poor survival. Ann Surg Oncol. 2009;16(1):200-7.
249. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2(2):127-37.
250. Roskoski R, Jr. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol Res. 2014;79:34-74.
251. Luo G, Long J, Qiu L, Liu C, Xu J, Yu X. Role of epidermal growth factor receptor expression on patient survival in pancreatic cancer: a meta-analysis. Pancreatology. 2011;11(6):595-600.
252. Tobita K, Kijima H, Dowaki S, Kashiwagi H, Ohtani Y, Oida Y, et al. Epidermal growth factor receptor expression in human pancreatic cancer: Significance for liver metastasis. Int J Mol Med. 2003;11(3):305-9.
253. Uegaki K, Nio Y, Inoue Y, Minari Y, Sato Y, Song MM, et al. Clinicopathological significance of epidermal growth factor and its receptor in human pancreatic cancer. Anticancer Res. 1997;17(5B):3841-7.
254. Bloomston M, Bhardwaj A, Ellison EC, Frankel WL. Epidermal growth factor receptor expression in pancreatic carcinoma using tissue microarray technique. Dig Surg. 2006;23(1-2):74-9.
255. Mikhitarian K, Pollen M, Zhao Z, Shyr Y, Merchant NB, Parikh A, et al. Epidermal growth factor receptor signaling pathway is frequently altered in ampullary carcinoma at protein and genetic levels. Mod Pathol. 2014;27(5):665-74.
256. Xia M, Overman MJ, Rashid A, Chatterjee D, Wang H, Katz MH, et al. Expression and clinical significance of epidermal growth factor receptor and insulin-like growth

factor receptor 1 in patients with ampullary adenocarcinoma. Hum Pathol. 2015;46(9):1315-22.
257. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015;372(8):724-34.
258. Aloysius MM, Lobo DN, Rowlands BJ, Madhusudan S, Ilyas M, Zaitoun AM. HER-2/Neu overexpression is a rare event in peri-ampullary cancer: assessment using the HercepTest. Histopathology. 2009;55(2):236-7.
259. Koeppen HK, Wright BD, Burt AD, Quirke P, McNicol AM, Dybdal NO, et al. Overexpression of HER2/neu in solid tumours: an immunohistochemical survey. Histopathology. 2001;38(2):96-104.
260. Safran H, Steinhoff M, Mangray S, Rathore R, King TC, Chai L, et al. Overexpression of the HER-2/neu oncogene in pancreatic adenocarcinoma. Am J Clin Oncol. 2001;24(5):496-9.
261. Chou A, Waddell N, Cowley MJ, Gill AJ, Chang DK, Patch AM, et al. Clinical and molecular characterization of HER2 amplified-pancreatic cancer. Genome Med. 2013;5(8):78.
262. Potti A, Ganti AK, Tendulkar K, Chitajallu S, Sholes K, Koch M, et al. HER-2/neu and CD117 (C-kit) overexpression in hepatocellular and pancreatic carcinoma. Anticancer Res. 2003;23(3B):2671-4.
263. Stoecklein NH, Luebke AM, Erbersdobler A, Knoefel WT, Schraut W, Verde PE, et al. Copy number of chromosome 17 but not HER2 amplification predicts clinical outcome of patients with pancreatic ductal adenocarcinoma. J Clin Oncol. 2004;22(23):4737-45.
264. Day JD, Digiuseppe JA, Yeo C, Lai-Goldman M, Anderson SM, Goodman SN, et al. Immunohistochemical evaluation of HER-2/neu expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasms. Hum Pathol. 1996;27(2):119-24.
265. Ata A, Polat A, Serinsoz E, Sungur MA, Arican A. Prognostic value of increased HER2 expression in cancers of pancreas and biliary tree. Pathol Oncol Res. 2015;21(3):831-8.
266. Komoto M, Nakata B, Amano R, Yamada N, Yashiro M, Ohira M, et al. HER2 overexpression correlates with survival after curative resection of pancreatic cancer. Cancer Sci. 2009;100(7):1243-7.
267. Koka V, Potti A, Koch M, Fraiman G, Mehdi S, Levitt R. Role of immunohistochemical identification of Her-2/neu and detection of variability in overexpression in pancreatic carcinoma. Anticancer Res. 2002;22(3):1593-7.
268. Safran H, Iannitti D, Ramanathan R, Schwartz JD, Steinhoff M, Nauman C, et al. Herceptin and gemcitabine for metastatic pancreatic cancers that overexpress HER-2/neu. Cancer Invest. 2004;22(5):706-12.
269. Hechtman JF, Liu W, Sadowska J, Zhen L, Borsu L, Arcila ME, et al. Sequencing of 279 cancer genes in ampullary carcinoma reveals trends relating to histologic subtypes and frequent amplification and overexpression of ERBB2 (HER2). Mod Pathol. 2015;28(8):1123-9.

270. Kawamoto T, Ishige K, Thomas M, Yamashita-Kashima Y, Shu S, Ishikura N, et al. Overexpression and gene amplification of EGFR, HER2, and HER3 in biliary tract carcinomas, and the possibility for therapy with the HER2-targeting antibody pertuzumab. J Gastroenterol. 2015;50(4):467-79.
271. O'Hayer K, Farber J, Yeo CJ, Sama AR. HER-2-Positive Ampullary Adenocarcinoma: A Case Report. Case Reports in Pancreatic Cancer. 2015;1(1):7-10.
272. Jaiswal BS, Kljavin NM, Stawiski EW, Chan E, Parikh C, Durinck S, et al. Oncogenic ERBB3 mutations in human cancers. Cancer Cell. 2013;23(5):603-17.
273. Fischer HP, Zhou H. Pathogenesis of carcinoma of the papilla of Vater. J Hepatobiliary Pancreat Surg. 2004;11(5):301-9.
274. Wang T, Liang YM, Hu P, Cheng YF. Mucins differently expressed in various ampullary adenocarcinomas. Diagn Pathol. 2011;6:102.
275. Allema JH, Reinders ME, van Gulik TM, Koelemay MJ, Van Leeuwen DJ, de Wit LT, et al. Prognostic factors for survival after pancreaticoduodenectomy for patients with carcinoma of the pancreatic head region. Cancer. 1995;75(8):2069-76.
276. van Roest MH, Gouw AS, Peeters PM, Porte RJ, Slooff MJ, Fidler V, et al. Results of pancreaticoduodenectomy in patients with periampullary adenocarcinoma: perineural growth more important prognostic factor than tumor localization. Ann Surg. 2008;248(1):97-103.
277. Eberhard J, Gaber A, Wangefjord S, Nodin B, Uhlen M, Ericson Lindquist K, et al. A cohort study of the prognostic and treatment predictive value of SATB2 expression in colorectal cancer. Br J Cancer. 2012;106(5):931-8.
278. Santini D, Vincenzi B, Fratto ME, Perrone G, Lai R, Catalano V, et al. Prognostic role of human equilibrative transporter 1 (hENT1) in patients with resected gastric cancer. J Cell Physiol. 2010;223(2):384-8.
279. Linderoth J, Ehinger M, Akerman M, Cavallin-Stahl E, Enblad G, Erlanson M, et al. Tissue microarray is inappropriate for analysis of BCL6 expression in diffuse large B-cell lymphoma. Eur J Haematol. 2007;79(2):146-9.

Paper I

Elebro and Jirström *Diagnostic Pathology* 2014, **9**:80
http://www.diagnosticpathology.org/content/9/1/80

DIAGNOSTIC PATHOLOGY

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Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma

Jacob Elebro[1,2*] and Karin Jirström[1]

Abstract

Background: Variability in reported histopathology parameters in operated periampullary adenocarcinomas may affect the prognostic weight of the parameters. Standardized axial sectioning produces a higher incidence of involved margins and also seems to produce a lower relative incidence of pancreatic compared with distal bile duct origin and a higher incidence of involved lymph nodes, compared with non-standardized procedure. The aims of this study were to 1) assess how a previously not described standardized pathology procedure, with longitudinal sectioning along the distal bile duct, affects reported tumour origin, margin status and involved lymph nodes, compared with non-standardized procedure, 2) assess if re-evaluation of microscopic slides affects the prognostic value of margin status and 3) compare the results of this standardized procedure with reported results of other standardized and non-standardized procedures.

Methods: One hundred seventy-five consecutive pancreaticoduodenectomy specimens with primary adenocarcinomas, operated during 2001 – 2011 at the University hospitals of Lund and Malmö, Sweden, were re-evaluated histologically, and parameters relevant for classification and prognosis were assessed, with 1 mm as a threshold for involved or uninvolved margins. Follow-up lasted until 31 December 2013. Five-year overall survival (OS) and hazard ratios (HR) were calculated for the margin status stated in the original reports and margin status after re-evaluation.

Results: Compared with non-standardized cases (n = 129), standardized cases (n = 46) had more involved lymph nodes in the specimens (median 3 vs 1), a higher fraction of distal bile duct origin (39% vs 21%) and a higher fraction of involved margins (74% vs 47%). The prognostic value of uninvolved margins increased by re-evaluation of slides ($p < 0.001$) and the adjusted HR for involved margins increased from 1.6 (95% CI 1.1 - 2.4) to 3.3 (95% CI 1.5 – 7.0). Uninvolved margins remained a significant predictor of OS in adjusted analysis.

Conclusions: Both the method of sectioning the specimen and the microscopic assessment affect prognostic pathology parameters significantly. The results of the herein described standardized method are similar to the results of other standardized procedures. The 1-mm threshold for involved margins in pancreaticoduodenectomies is relevant for OS, and margin status is an independent prognostic parameter.

Virtual slides: The virtual slides for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/1056639379120615

Keywords: Carcinoma, Pancreatic ductal, Common bile duct neoplasms, Duodenal neoplasms, Pathology, Surgical, Pancreaticoduodenectomy, Prognosis

* Correspondence: Jacob.Elebro@med.lu.se
[1]Department of Clinical Sciences, Division of Oncology and Pathology, Lund University, Skåne University Hospital, Lund SE-221 85, Sweden
[2]Skåne University and Regional Laboratories, Lund, Sweden

Background

Pathology guidelines that change the incidence of histopathology parameters are clinically relevant since the parameters carry prognostic information. Guidelines on gross examination and sectioning of pancreaticoduodenectomy (PD) specimens have changed during the last years, after the introduction of the Leeds pathology protocol (LEEPP) [1]. This standardized procedure raised the incidence of involved margins (R1) and involved lymph nodes (N1), and also decreased pancreatic origin and increased distal bile duct origin [2,3] compared to large series using non-standardized procedures [4-10].

Proportions of tumour origin vary greatly between different series of operated periampullary adenocarcinomas and it is not known which proportions most accurately reflect the biology of the tumours, or are most clinically relevant. It is however evident that a meticulous pathology examination improves the quality of the pathology report for these cancer forms by producing a higher incidence of N1 and R1 [2]. A high proportion of R1 also seems to correlate to a low relative incidence of pancreatic origin, suggesting that a more thorough examination decreases the relative incidence of pancreatic origin [11]. So far, the reported increase of R1 and decrease in pancreatic origin in the LEEPP-series has been attributed to this particular slicing method. It is however not clear to what extent this change is due to the method or to the interest and dedication of the pathologist.

Here, we present the results of a different standardized protocol (SP), in which the pathologist gains access to the full length of the common bile duct through a longitudinal opening via the posterior margin of the PD-specimen, and only standard size blocks are made. It has been stated that this method is inferior to the LEEPP, due to its limited value for assessing tumour origin and resection margins [1]. This method has however not been studied in a standardized setting before.

Methods

Data collection and patient characteristics

The study cohort is a retrospective consecutive series of 175 PD-specimens with primary adenocarcinomas surgically treated at the University hospitals of Lund and Malmö, Sweden, from January 1 2001 until December 31 2011. Data on survival were gathered from the Swedish National Civil Register. Follow-up started at the date of surgery and ended at death or at December 31 2013, whichever came first.

Data on margin status was collected from the original pathology reports, as were data on age at surgery, date of surgery, sex, and whether the specimen was handled according to the SP or not. Data was also gathered on the origin of lymph nodes submitted in separate containers. After information was given on how and from where the surgeons harvested lymph nodes submitted in separate containers, positions 6, 8, 12, 13, 14 and 17 were classified as originating from the specimen, and other positions including 9 and 16 were classified as not originating from the specimen.

Of the 175 PDs, 46 (26%) were examined and sectioned according to our SP by one pathologist (JE) and 129 (74%) were examined and sectioned by several pathologists according to personal choice (non-standardized protocol, NSP).

Ethical permission was obtained from the Ethics Committee at Lund University.

Sectioning of the specimens, standardized protocol

This method is, by opening the PD-specimen along the bile duct, similar to one of the methods earlier described by the Royal College of Pathologists [12] but performed in a standardized manner and without opening the pancreatic duct.

The specimens were handled after fixation in formalin (Figure 1). Margins were stained in different colours; one for the pancreatic transection margin, one for the margin towards the superior mesenteric vein (SMV), one for the margin towards the superior mesenteric artery (SMA), one for the anterior surface and one for the posterior margin. The specimens were accessed through a longitudinal opening of the common bile duct at the posterior margin, from the most proximal part of the bile duct through the papilla of Vater. In the same plane the section was deepened through the common bile duct and into the pancreatic parenchyme. This produced a book-like opening that visualized the whole length of the common bile duct, the ampulla and adjacent pancreatic parenchyme as well as parts of the posterior margin and parts of the SMV-margin. Several standard size blocks were sampled from the ampulla with adjacent duodenal mucosa, pancreatic parenchyme and anterior and posterior margins. The bile duct was sampled longitudinally, with adjacent pancreatic parenchyme, posterior margin and SMV-margin. Additional standard size blocks were sampled from the SMA-margin, from all visible or palpable lymph nodes in the specimen and from additional areas with possible tumour growth. En face sections were made from the pancreatic, bile duct, pyloric and duodenal transection margins.

Standardized protocol vs non-standardized protocol

Re-evaluations of slides

All haematoxylin & eosin stained slides from all cases were revised by one pathologist (JE), blinded to the original report and outcome. Other stains were not revised or used for the assessment of any parameter. Data were gathered on tumour origin, size and grade, perineural invasion, lymphatic vessel and blood vessel invasion, invasion

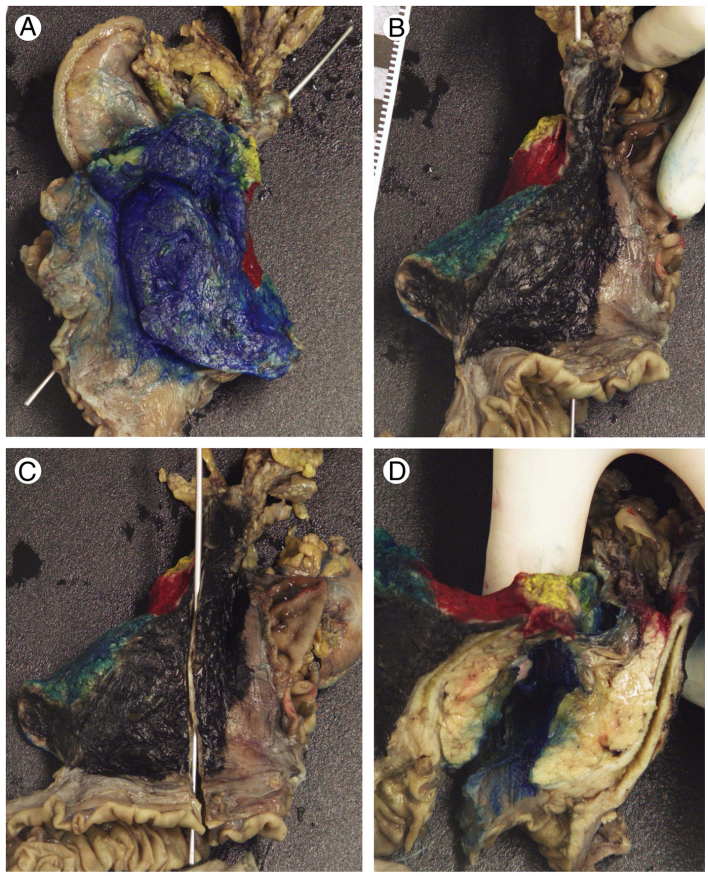

Figure 1 Accessing a pancreaticoduodenectomy specimen through the bile duct. (A) Anterior view of a painted pancreaticoduodenectomy specimen. Posterior – black, anterior – blue, pancreatic transection margin – yellow, SMV-margin - red and SMA-margin - green. A probe is inserted in the lumen of the bile duct. **(B)** posterior view, **(C)** posterior view with the bile duct opened longitudinally, and **(D)** the pancreatic parenchyme accessed through the bile duct, visualizing the ampulla, the bile duct and parts of the pancreatic parenchyme, as well as parts of the margins.

of peripancreatic fat, number of lymph nodes and involved lymph nodes found by the pathologist in the specimen, number of lymph nodes and involved lymph nodes harvested from the specimen by the surgeon and submitted in separate containers, number of lymph nodes and involved lymph nodes in separate containers originating from other areas, N-stage, T-stage and margin status.

Decision on tumour origin was based on the anatomical centre of the tumour, with the aid of preinvasive precursor lesions or multifocality, if present. A tumour in the duodenal mucosa with intestinal morphology that involved the ampulla in the periphery was considered to be of duodenal origin. A similar tumour with the ampulla in the centre was considered to be of ampullary origin. A tumour along the bile duct that involved the ampulla was considered to be of bile duct origin if the ampulla was in the periphery of the tumour, but of ampullary origin if the ampulla was in the centre. Multifocal tumour growth or multifocal premalignant changes in the pancreatic parenchyme in the absence of evidence of other tumour origin was considered as a sign of pancreatic origin. In addition to tumour origin the distinction between intestinal morphology and

pancreaticobiliary morphology was made for all ampullary carcinomas using morphological criteria [13].

For the assessment of tumour grade, only the poorest degree of differentiation was recorded.

Margin status was denoted as R1 if cancer was present less than 1 mm from any margin except for the duodenal serosa, as R0 if the shortest distance exceeded 1 mm, and as unknown (Rx) if any margin, except the duodenal serosa close to the cancer, was insufficiently sampled. If a margin was considered sufficiently sampled or not differed by the location of the tumour. In addition to pancreatic and distal bile duct transection margins, an ampullary carcinoma needed at least one standard size block showing the relation to the anterior surface, adjacent to the duodenal wall, two showing the relation to the posterior surface adjacent to the duodenal wall, one from the SMA-margin and one from the SMV-margin, in order to be considered sufficiently sampled regarding margins. Carcinomas of pancreatic or distal bile duct origin needed, in addition to pancreatic and distal bile duct transection margins, at least two blocks showing the relation to the posterior margin, one from the SMA-margin, one from the SMV-margin and one from the anterior margin. For duodenal origin, one block each from the posterior and anterior margins adjacent to the duodenal wall was considered sufficient. A case could be considered as R1 in an unspecified margin even if other margins were insufficiently sampled.

For sampling of lymph nodes in the specimen, the full surface around the specimens was searched manually and also visually after sectioning in intervals of approximately 3 mm.

Statistical analysis

The Chi-square test and Fisher's Exact test were used to analyse differences in the distribution of histopathological factors in relation to use of standardized vs non-standardized protocol, and according to tumour location. Kaplan-Meier analysis and log rank test were used to illustrate differences in 5-year overall survival (OS) in strata according to margin status. Cox regression models were used to calculate hazard ratios (HR) for the impact of histopathology parameters on 5-year OS, in univariable and multivariable analysis, adjusted for age, sex, tumour morphology, tumour size, tumour grade, T-stage, N-stage, margin status, perineural invasion, growth in peripancreatic fat, invasion of lymphatic vessels and invasion of blood vessels. Cases who died within 1 month from surgery (n = 2) or were lost to follow up (n = 1) were excluded from the survival analyses.

All tests were two-sided and a p-value <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

The annual PD-rate increased during the study period, with 35 and 29 cases operated in 2010 and 2011, respectively, compared to a median of 13 per year (range 8–19) during 2001–2009. Forty-two of the 46 SP-cases were diagnosed during 2010 – 2011, which coincided with an increased number of lymph nodes sent for analysis in separate containers; median 1 (interquartile range, IQR 0 – 2) during 2001 – 2009 and median 7 (IQR 3.25 – 10) during 2010 – 2011.

Median 5-year OS was 30.4 months in the full cohort of all 172 SP- and NSP-cases, 35.0 months in the SP-group and 29.7 months in the NSP-group. In the SP-group of 46 cases, 27 died during follow up and 19 were censored at December 31 2013. Out of the 129 NSP-cases, 3 were excluded from the survival analysis, but included in all other analyses. Of the remaining 126 cases, 88 died during follow up and 38 were censored at December 31 2013.

Differences in the distribution of histopathological parameters between SP-cases and NSP-cases

As shown in Table 1, there were several significant differences in the distribution of histopathological parameters between the re-evaluated NSP- and SP-materials.

Tumour origin differed between the SP-group and the NSP-group (p = 0.040), with a higher proportion of distal bile duct origin (39% vs 21%) and a lower proportion of ampullary origin (26% vs 45%) in the former.

There was no significant difference between the SP-group and the NSP-group regarding the number of lymph nodes found by the pathologist in the PD-specimens, but the number of lymph nodes harvested from the specimen by the surgeon, as well as the total number of lymph nodes originating from the PD-specimens, was significantly higher in the SP-group compared with the NSP-group (p < 0.001 for both).

The number of involved lymph nodes in the PD-specimens was also significantly higher in the SP-group as compared with the NSP-group (p = 0.001), and the number of involved lymph nodes from the PD-specimens submitted in separate containers and total number of involved lymph nodes originating from the specimens differed significantly. The proportion of cases with involved lymph nodes (N1-N2) did not differ significantly between the SP-group and NSP-group.

Since the increase in the number of lymph nodes harvested from the specimen by the surgeon occurred in 2009, a separate analysis on lymph node-variables was performed for the last 2.5 years of the study period (July 2009 – 2011). This revealed a significant difference between the SP-group (n = 44) and the NSP-group (n = 31) in the number of involved lymph nodes found in the PD-specimens by the pathologist (median 2.5 vs 1, p = 0.046). There were however no significant differences in the total

Table 1 Standardized vs non-standardized protocol: Characteristics of 175 re-evaluated periampullary adenocarcinomas

	NSP (n = 129)	SP (n = 46)	p-value	All (n = 175)
Tumour origin			**0.040**	
Duodenum	9 (7%)	5 (11%)		14 (8%)
Ampulla, both types	58 (45%)	12 (26%)		70 (40%)
Distal Bile Duct	27 (21%)	18 (39%)		45 (26%)
Pancreas	35 (27%)	11 (24%)		46 (26%)
Tumour size, mm				
M (IQR)	30 (20–35)	30 (25–40)	0.649	30 (21–35)
Larger than 20 mm	92 (71%)	42 (91%)	**0.008**	134 (77%)
Differentiation, poor	70 (54%)	31 (67%)	0.164	101 (58%)
Lymph nodes				
In PD specimen, M (IQR)	6 (3–10)	9 (7–13)	0.102	7 (4–10)
From PD specimens, M (IQR)	1 (0–2)	7 (3–9)	**<0.001**	2 (0–5)
Total PD specimen, M (IQR)	8 (5–12)	16 (12–19)	**<0.001**	11 (6–15)
≥10 lymph nodes PD specimen, n (%)	58 (45%)	40 (87%)	**<0.001**	98 (56%)
Other local lymph nodes, M (IQR)	0 (0–1)	0 (0–1)	0.400	0 (0–1)
Total, all lymph nodes, M (IQR)	9 (5–13)	16 (13–20)	**<0.001**	11 (6–16)
Involved lymph nodes				
In PD specimen, M (IQR)	1 (0–2)	3 (0–4)	**0.001**	1 (0–3)
From PD specimen, M (IQR)	0 (0–0)	0 (0–1)	**0.024**	0 (0–0)
Total PD specimen, M (IQR)	1 (0–2)	3 (0–4)	**0.023**	1 (0–3)
Other local, M (IQR)	0 (0–0)	0 (0–0)	**0.017**	0 (0–0)
Total, all involved lymph nodes, M (IQR)	1 (0–2)	3 (0–4)	**0.015**	1 (0–3)
N-stage, pN1 (for duodenum pN1-N2)	74 (57%)	33 (72%)	0.113	107 (61%)
Margin involvement			**<0.001**	
R1	60 (47%)	34 (74%)		94 (54%)
Rx (uncertain/unassessable)	56 (43%)	0 (0%)		56 (32%)
R0	13 (10%)	12 (26%)		25 (14%)
Perineural infiltration	71 (55%)	34 (74%)	**0.035**	105 (60%)
Infiltration in lymph vessels	79 (61%)	32 (70%)	0.374	111 (63%)
Infiltration in blood vessels	38 (29%)	4 (9%)	**0.004**	42 (24%)
Infiltration in peripancreatic fat	71 (55%)	36 (78%)	**0.008**	107 (61%)
T-stage			0.074	
pT1	8 (6%)	0 (0%)		8 (4%)
pT2	21 (17%)	3 (6%)		24 (14%)
pT3	70 (54%)	33 (72%)		103 (59%)
pT4	30 (23%)	10 (22%)		40 (23%)
Blocks from PD-specimen				
Regular blocks, Median (IQR)	15 (12–22)	23 (20–27)	**0.001**	
Mean (min - max)	17 (6–48)	24 (14–36)		
Large blocks, Median (IQR)	0 (0–2)	0 (0–0)	**<0.001**	
Mean (min - max)	1 (0–8)	0 (0–0)		

SP, standardized procedure. NSP, non-standardized procedure. PD, pancreatoduodenectomy. M, median. IQR, interquartile range. SMA, superior mesenteric artery. SMV, superior mesenteric vein. For margin involvement p-values were calculated R1 vs R0 and Rx. Bold text indicates $p < 0.05$.

number of lymph nodes from the specimen (median 16 vs 12, p = 0.601), fraction of cases with 10 or more lymph nodes (89% vs 74%, p = 0.128) or fraction of cases with involved lymph nodes (71% vs 65%, p = 0.622).

As further shown in Table 1, there was a significantly larger proportion of R1 cases (p = 0.002), tumours larger than 20 mm (p = 0.008), perineural tumour growth (p = 0.035) and infiltration of peripancreatic fat (p = 0.002) in the SP-group compared with the NSP-group. In contrast, infiltration of blood vessels was more often found in the NSP-group (p = 0.004).

We also examined the involvement of different resection margins by tumour type (Table 2). Significant differences (R0 vs R1 and Rx) between the SP and non-SP groups were found at the posterior margin (p = 0.001), the SMA-margin (p < 0.001) and the SMV-margin (p < 0.001), and in tumours of distal bile duct origin (p = 0.006).

Effect of re-evaluations of slides

The distribution of histopathological characteristics in the total re-evaluated material, stratified by tumour origin, is shown in Table 3. In the original reports there were 14 NSP-cases without information on margin status. Re-evaluation of slides changed margin status for the NSP-group, increasing R1 from 45/115 to 60/129 and decreasing R0 from 70/115 to 12/129 (p < 0.001), and re-evaluations also rendered 56 NSP-cases with unknown margin status (Rx). Re-evaluation of slides rendered a non-significant increase of R1 in the SP-material, from 63% (29/46) to 76% (35/46) (p = 0.257).

Re-evaluations revealed lymph node involvement in 20% (14/70) of NSP-cases that were N0 in the original report. This caused a non-significant change in fraction with involved lymph nodes in the NSP-group, from 46% (59/129) to 57% (73/129) (p = 0.105). Re-evaluations rendered no alterations in the fraction of involved lymph nodes in the SP-material.

Overall survival in relation to margin status

Kaplan-Meier analysis revealed a significantly prolonged five-year OS in the re-evaluated R0-group compared with the original report R0-group (p < 0.001) (Figure 2). As further shown in Table 4, the unadjusted HR for R1 vs R0 in the original report was 1.6 (95% CI 1.1 - 2.4). In the re-evaluated material the unadjusted HR for R1 vs R0 was 3.3 (95% CI 1.5 - 7.0) and the unadjusted HR for Rx vs R0 was 2.3 (95% CI 1.0 - 5.2). Re-evaluated, but not originally reported, margin status remained an independent prognostic factor in adjusted analysis (HR 2.2, 95% CI 1.0 - 4.9 for R1 and Rx vs R0) (Table 4). The unadjusted and adjusted HRs for re-evaluated histopathology parameters are shown in Table 5.

Discussion

This is, to our best knowledge, the first report on standardized longitudinal opening and slicing of the common bile duct in the handling of PD-specimens with primary adenocarcinoma.

Our results confirm previous reports on standardized protocols in the pathology examination of operated periampullary adenocarcinomas by showing that a 1-mm cut-off in the assessment of margin status is relevant for overall survival, both in unadjusted analysis and after adjusting for other histopathology parameters. Microscopic re-evaluation of margin status revealed a larger proportion of involved margins than stated in the original reports. Thereby, the prognostic value of uninvolved margins was increased, regardless of other histopathology

Table 2 Margin status and tumour origin

	Duodenum		Ampulla		Distal Bile Duct		Pancreas		All tumour origins		
	NSP	SP	NSP	SP	NSP	SP	NSP	SP	NSP	SP	p-value
	9	5	58	12	27	18	35	11	129	46	
R1, n (%)	1 (11%)	2 (40%)	19 (33%)	5 (42%)	15 (56%)	17 (94%)	25 (71%)	10 (91%)	60 (47%)	34 (74%)	**0.002**
R0, n (%)	2 (22%)	3 (60%)	7 (12%)	7 (58%)	3 (11%)	1 (6%)	1 (3%)	1 (9%)	13 (10%)	12 (26%)	
Rx, n (%)	6 (67%)	0	32 (55%)	0	9 (33%)	0	9 (26%)	0	56 (43%)	0	
Pancreas transection margin	0	1	2	0	3	2	9	1	14 (11%)	4 (9%)	0.784
DBD transection margin	0	0	0	0	1	1	1	0	2 (2%)	1 (2%)	1.000
SMA margin	0	0	0	0	2	8	0	2	2 (2%)	10 (22%)	**<0.001**
Posterior surface	0	2	8	4	7	10	7	4	22 (17%)	20 (44%)	**0.001**
SMV surface	0	1	0	0	3	10	10	8	13 (10%)	19 (41%)	**<0.001**
Anterior surface	0	1	1	2	4	1	3	2	8 (6%)	6 (13%)	0.202

Margin status in 175 re-evaluated pancreaticoduodenectomies. NSP, non-standardized protocol. SP, standardized protocol. DBD, distal bile duct. SMA, superior mesenteric artery. SMV, superior mesenteric vein. For percentages and significances, calculations were made R0 vs R1 and Rx. In separate tumour origins, differences in R1-fraction between the SP-group and the NSP-group were significant in distal bile duct origin, (p = 0.006). Some NSP-cases were classified as R1 in an unspecified margin. R1-cases could have more than one involved margin. Bold text indicates p < 0.05.

Table 3 Distribution of clinicopathological characteristics according to tumour origin in 175 re-evaluated periampullary adenocarcinomas

	Duodenum	Ampulla Intestinal type	Ampulla Pancreatobiliary type	Distal Bile Duct	Pancreas	All
	n = 14	n = 51	n = 19	n = 45	n = 46	n = 175
Standardized Procedure	5 (36%)	7 (14%)	5 (26%)	18 (40%)	11 (24%)	46 (26%)
Age at surgery, M (IQR)	68 (62 – 74)	67 (59 – 70)	69 (62 – 75)	64 (59 – 71)	68 (62 – 72)	67 (61 – 72)
Gender, Female	6 (43%)	29 (57%)	9 (47%)	21 (47%)	21 (46%)	86 (49%)
Tumour size, mm, M (IQR)	40 (30 – 53)	23 (15–30)	30 (24 – 40)	26 (22 – 35)	30 (25 – 35)	30 (21 – 35)
Larger than 20 mm	13 (93%)	27 (53%)	18 (95%)	37 (82%)	39 (85%)	134 (77%)
High grade	7 (50%)	26 (51%)	9 (47%)	31 (69%)	28 (61%)	101 (58%)
Lymph nodes, M (IQR)	10 (6 – 13.5)	9 (6 – 16)	10 (5 – 17)	12 (8 – 16.5)	11.5 (6.75 – 16)	11 (6 – 16)
10 or more lymph nodes	8 (57%)	25 (49%)	10 (53%)	30 (67%)	27 (59%)	100 (57%)
Involved lymph nodes, M (IQR)	0 (0 – 2)	0 (0 – 2)	2 (1 – 7)	1 (0 – 4)	2 (0 – 3)	1 (0 – 3)
pN1	4 (29%)	24 (47%)	16 (84%)	27 (60%)	34 (74%)	106 (61%)
pN2	2 (14%)					
Perineural infiltration	4 (29%)	16 (31%)	14 (74%)	37 (82%)	34 (74%)	105 (60%)
Infiltration in lymph vessels	2 (14%)	34 (67%)	15 (79%)	33 (73%)	27 (59%)	111 (63%)
Infiltration in blood vessels	0 (0.0%)	5 (10%)	8 (42%)	14 (31%)	15 (33%)	42 (24%)
Infiltration in peripancreatic fat	6 (43%)	16 (31%)	17 (89%)	36 (80%)	32 (70%)	107 (61%)
T-stage (pTNM)						
T1	0	5 (10%)	0	1 (2%)	2 (4%)	
T2	1 (7%)	11 (22%)	0	2 (4%)	10 (22%)	
T3	6 (43%)	19 (37%)	2 (11%)	42 (93%)	34 (74%)	
T4	7 (50%)	16 (31%)	17 (89%)	0	0	
R1	3 (38%)	10 (43%)	14 (93%)	32 (89%)	35 (95%)	94 (79%)
R0	5 (63%)	13 (57%)	1 (7%)	4 (11%)	2 (6%)	25 (21%)
Rx,uncertain margin status (n)	6	28	4	9	9	56
Pancreatic transection margin	1 (13%)	1 (4%)	1 (7%)	5 (14%)	10 (27%)	18 (15%)
DBD transection margin	0	0	0	2 (6%)	1 (3%)	3 (3%)
SMA-margin	0	0	0	10 (28%)	2 (5%)	12 (10%)
Posterior margin	2 (25%)	6 (26%)	6 (40%)	17 (47%)	11 (30%)	42 (35%)
SMV-margin	1 (13%)	0	0	13 (36%)	18 (49%)	32 (27%)
Anterior margin	1 (13%)	1 (4%)	2 (13%)	5 (14%)	5 (14%)	14 (12%)
5-year OS, M (IQR), months	n.r. (37 - n.r.)	53 (26 - n.r.)	26 (15–40)	25 (16 - n.r.)	25 (13–42)	30 (17-n.r.)

M, median. IQR, interquartile range. OS, overall survival. N.r., not reached.

parameters. This suggests that a "guilty until proven innocent"-approach towards margins in pancreaticoduodenectomies gives more accurate prognostic information than the opposite approach. Moreover, survival in the large group of cases with unassessable margin status (Rx) differed significantly both from cases with uninvolved margins and from cases with involved margins, suggesting that it is not appropriate to classify these cases as R0.

The more frequent finding of growth in peripancreatic fat and perineural tumour growth in SP-cases compared to NSP-cases may be an effect of more extensive sampling in the periphery of the tumour as well as along the bile duct and margins in SP-cases compared with NSP-cases.

Tumour infiltration in blood vessels was more often found in NSP-cases than in SP-cases (29% vs 9%), which may be due to an unintended more thorough search for evaluable pathology parameters in SP-cases that had very little coverage on margins and lymph nodes. This model of explanation suggests that the proportion of cases with tumour infiltration in blood vessels in the NSP-group more accurately reflects the actual percentage of infiltration in blood vessels. As a cautionary remark, the

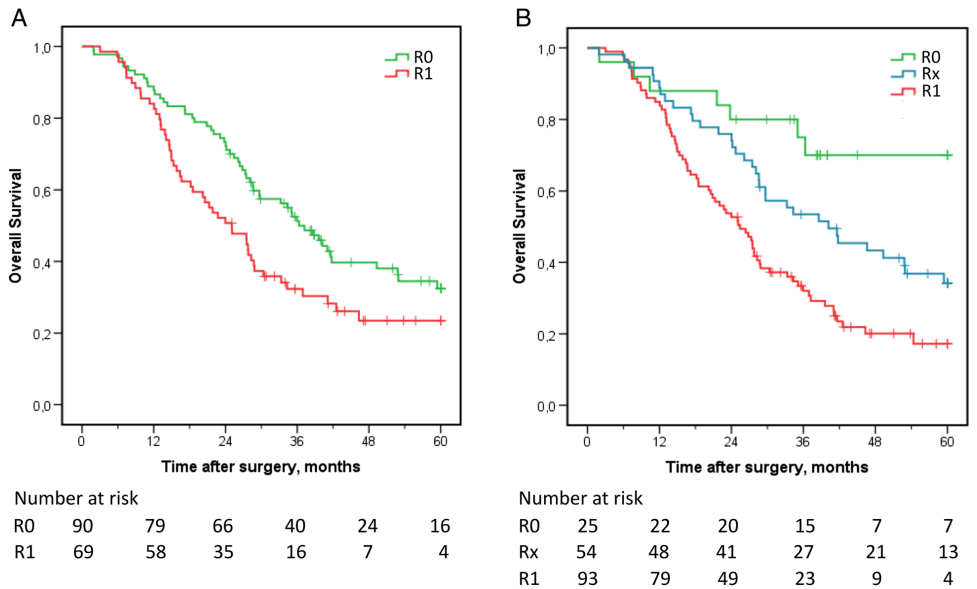

Figure 2 Kaplan-Meier estimates of five-year overall survival in relation to margin status before and after re-evaluation. Five-year OS in relation to **(A)** margin status stated in the original reports and **(B)** margin status upon re-evaluation of slides.

possibility of a type I error, i.e. a false positive detection of significant differences between the NSP-group and the SP-group, should also be considered, since a large number of comparisons have been performed. A type II error, i.e. failure to detect the true incidence of involved blood vessels in the SP-group, is also possible due to the relatively small sample size in this group.

Comparisons of the incidence of involved margins between our SP-material, excluding duodenal origin, and other standardized series show 78% R1 (32/41) in our SP-group compared with 59% (32/54) and 61% (51/83) in the LEEPP-series [2,3]. The incidence of involved margins is often not comparable between SP-series and NSP-series, due to a 0-mm definition of margin involvement, or lack of definitions on margin involvement in NSP-series. The fraction of cases with involved lymph nodes is however comparable, showing that non-standardized series [4-10] report involved lymph nodes in less than 60% of cases, compared to more than 70% in our SP-group and in the LEEPP-series. If such differences are coincidental or actually statistically significant, as well as their potential clinical significance, remains unknown. In the present study, we were able to demonstrate a significantly higher number of involved lymph nodes in the specimens in the SP-group compared with the NSP-group, despite a temporal association between an increased number of lymph nodes

Table 4 Five year overall survival in relation to margin status in the original reports and after re-evaluation

	Margin status in original reports				Margin status after re-evaluation			
	Number	Median OS (IQR)	Unadjusted HR (95% CI)	Adjusted HR (95% CI)	Number	Median OS (IQR)	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
R0	90	36.3 (23.1 - n.r.)			25	n.r. (35.0 - n.r.)		
Rx					54	40.2 (24.0 - n.r.)	2.3 (1.0 - 5.2)	2.2 (1.0 - 4.9)
R1	69	25.1 (14.0 - 46.3)	1.6 (1.1 - 2.4)	1.0 (0.7 - 1.6)	93	25.4 (14.6 - 41.6)	3.3 (1.5 - 7.0)	

Hazard ratios (HR) for risk of death within 5 years in relation to margin status, with R0 as reference. OS in the re-evaluated R0-group was significantly better than in the original report R0-group ($p < 0.001$). Differences in OS between the three levels of margin status in the re-evaluated material were significant; R0 vs R1 ($p < 0.001$), R1 vs Rx ($p = 0.005$) and R0 vs Rx ($p = 0.043$). HR for both original report and re-evaluated margin status adjusted for re-evaluated parameters. R0, uninvolved margins. Rx, uncertain margin status. R1, cancer less than 1 mm from margin. IQR, interquartile range. CI, confidence interval. N.r., not reached.

Table 5 Unadjusted and adjusted hazard ratios for death within 5 years in relation to re-evaluated histopathology parameters

	n (events)	Unadjusted HR (95% CI)	p-value	Adjusted HR (95% CI)	p-value
Age, continuous	172 (112)	1.0 (1.0-1.0)	0.479	1.0 (1.0-1.1)	**0.015**
Sex					
Female	86 (47)				
Male	86 (65)	1.5 (1.0-2.2)	**0.042**	1.4 (0.9-2.0)	0.131
T-stage					
T1	8 (3)				
T2	23 (12)	1.5 (0.4-5.2)	0.553	1.1 (0.3-4.4)	0.847
T3	102 (66)	2.7 (0.8-8.6)	0.995	1.0 (0.3-3.8)	0.948
T4	39 (31)	3.4 (1.0-11.2)	**0.043**	1.3 (0.3-5.1)	0.683
N-stage					
N0	67 (37)				
N1-N2	105 (75)	2.0 (1.3-2.9)	**0.001**	1.3 (0.8-2.0)	0.265
Tumour size, continuous	172 (112)	1.0 (1.0-1.0)	**0.010**	1.0 (1.0-1.0)	0.533
Tumour differentiation					
Well-moderate	73 (38)				
Poor	99 (74)	2.3 (1.5-3.3)	**<0.001**	1.9 (1.2-2.8)	**0.002**
Tumour morphology					
Intestinal type	63 (31)				
Pancreatobiliary type	109 (81)	2.3 (1.5-3.4)	**<0.001**	1.3 (0.7-2.3)	0.394
Margins					
R0	25 (7)				
R1-Rx	147 (105)	3.3 (1.5-7.0)	**0.002**	2.2 (1.0-4.9)	**0.046**
Perineural growth					
No	68 (33)				
Yes	104 (79)	2.4 (1.6-3.7)	**<0.001**	1.1 (0.6-1.8)	0.779
Growth in lymphatic vessels					
No	63 (30)				
Yes	109 (82)	2.2 (1.4-3.3)	**<0.001**	1.2 (0.8-1.9)	0.420
Growth in blood vessels					
No	131 (74)				
Yes	41 (38)	3.1 (2.1-4.7)	**<0.001**	2.4 (1.6-3.7)	**<0.001**
Growth in peripancreatic fat					
No	67 (30)				
Yes	105 (82)	2.8 (1.8-4.3)	**<0.001**	2.1 (1.4-3.3)	**0.001**

HR, hazard ratio. CI, confidence interval. Bold text indicates $p < 0.05$.

harvested from the specimens by the surgeons and the studied standardized protocol.

In our material the differences in tumour origin between the SP-group and the NSP-group were significant. It is however not known if there are any clinically relevant differences between the tumour origins of standardized and non-standardized series. It has however previous been shown that the morphological distinction between intestinal and pancreatobiliary morphology has prognostic implications, not only in ampullary adenocarcinomas, but in all periampullary adenocarcinomas, regardless of tumour origin [14]. Moreover, while differences in the expression of cytokeratins and mucins according to morphology have been observed in ampullary carcinomas [15], these differences seem to be less evident in series stratified solely by the anatomical centre of the ampullary adenocarcinomas [16]. These findings suggest that morphological and molecular tumour characteristics have a

greater prognostic impact than the appreciated tumour origin.

Despite a very different approach to the specimen, the results on tumour origin, N-stage and margin status in our standardized group are similar to the results of the LEEPP-series [2,3] and to a lesser degree similar to the results of two other variants on standardized protocols [17,18]. Whether or not our standardized protocol was more time consuming or more demanding than the LEEPP, and thus inferior due to practical reasons, has however not been studied.

Conclusions

A 1-mm threshold for margin involvement is relevant for overall survival in operated periampullary adenocarcinomas, regardless of tumour origin and other histopathology parameters. Standardized protocols on sectioning of pancreaticoduodenectomy specimens seem to increase the yield of adverse prognostic histopathology parameters compared with non-standardized protocols. Standardizations in pancreatic pathology are needed to decrease unjustifiable variability in pathology reports, both for the sake of the treatment of individual patients and for the sake of future studies and clinical trials.

Abbreviations

OS: Overall survival; HR: Hazard ratio; PD: Pancreaticoduodenectomy; LEEPP: Leeds pathology protocol; R1: Involved margins; Rx: Unknown margin status; R0: Uninvolved margins; N1-N2: Involved lymph nodes; SP: Standardized protocol; NSP: Non-standardized protocol; SMV: Superior mesenteric vein; SMA: Superior mesenteric artery; M: Median; IQR: Interquartile range; T-stage: Tumour stage; N-stage: Lymph node stage.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JE conceived of the study, collected data, performed the statistical analyses and drafted the manuscript. KJ participated in the design of the study, statistical analyses and drafting of the manuscript. Both authors read and approved the final manuscript.

Acknowledgments

This study was supported by grants from the Knut and Alice Wallenberg Foundation, the Swedish Cancer Society, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and University Hospital Research Grants.

Received: 5 February 2014 Accepted: 1 April 2014
Published: 14 April 2014

References

1. Verbeke CS: **Resection margins and R1 rates in pancreatic cancer–are we there yet?** *Histopathology* 2008, **52:**787–796.
2. Verbeke CS, Leitch D, Menon KV, McMahon MJ, Guillou PJ, Anthoney A: **Redefining the R1 resection in pancreatic cancer.** *Br J Surg* 2006, **93:**1232–1237.
3. Menon KV, Gomez D, Smith AM, Anthoney A, Verbeke CS: **Impact of margin status on survival following pancreatoduodenectomy for cancer: the Leeds Pathology Protocol (LEEPP).** *HPB (Oxford)* 2009, **11:**18–24.
4. Hatzaras I, George N, Muscarella P, Melvin WS, Ellison EC, Bloomston M: **Predictors of survival in periampullary cancers following pancreaticoduodenectomy.** *Ann Surg Oncol* 2010, **17:**991–997.
5. Jarufe NP, Coldham C, Mayer AD, Mirza DF, Buckels JA, Bramhall SR: **Favourable prognostic factors in a large UK experience of adenocarcinoma of the head of the pancreas and periampullary region.** *Dig Surg* 2004, **21:**202–209.
6. Allema JH, Reinders ME, van Gulik TM, Koelemay MJ, Van Leeuwen DJ, de Wit LT, Gouma DJ, Obertop H: **Prognostic factors for survival after pancreaticoduodenectomy for patients with carcinoma of the pancreatic head region.** *Cancer* 2069–2076, **1995:**75.
7. Riall TS, Cameron JL, Lillemoe KD, Winter JM, Campbell KA, Hruban RH, Chang D, Yeo CJ: **Resected periampullary adenocarcinoma: 5-year survivors and their 6- to 10-year follow-up.** *Surgery* 2006, **140:**764–772.
8. van Roest MH, Gouw AS, Peeters PM, Porte RJ, Slooff MJ, Fidler V, de Jong KP: **Results of pancreaticoduodenectomy in patients with periampullary adenocarcinoma: perineural growth more important prognostic factor than tumor localization.** *Ann Surg* 2008, **248:**97–103.
9. van Geenen RC, van Gulik TM, Offerhaus GJ, de Wit LT, Busch OR, Obertop H, Gouma DJ: **Survival after pancreaticoduodenectomy for periampullary adenocarcinoma: an update.** *Eur J Surg Oncol* 2001, **27:**549–557.
10. Schmidt CM, Powell ES, Yiannoutsos CT, Howard TJ, Wiebke EA, Wiesenauer CA, Baumgardner JA, Cummings OW, Jacobson LE, Broadie TA, Canal DF, Goulet RJ Jr, Curie EA, Cardenes H, Watkins JM, Loehrer PJ, Lillemoe KD, Madura JA: **Pancreaticoduodenectomy: a 20-year experience in 516 patients.** *Arch Surg* 2004, **139:**718–727.
11. Verbeke CS, Gladhaug IP: **Resection margin involvement and tumour origin in pancreatic head cancer.** *Br J Surg* 2012, **99:**1036–1049.
12. Campbell F, Bennett MK, Foulis AK: **Standards and minimum datasets for reporting cancers. Minimum dataset for the histopathological reporting of pancreatic, ampulla of Vater and bile duct carcinoma.** London: The Royal College of Pathologists; 2002.
13. Kimura W, Futakawa N, Yamagata S, Wada Y, Kuroda A, Muto T, Esaki Y: **Different clinicopathologic findings in two histologic types of carcinoma of papilla of Vater.** *Jpn J Cancer Res* 1994, **85:**161–166.
14. Westgaard A, Tafjord S, Farstad IN, Cvancarova M, Eide TJ, Mathisen O, Clausen OP, Gladhaug IP: **Pancreatobiliary versus intestinal histologic type of differentiation is an independent prognostic factor in resected periampullary adenocarcinoma.** *BMC cancer* 2008, **8:**170.
15. Fischer HP, Zhou H: **Pathogenesis of carcinoma of the papilla of Vater.** *J Hepatobiliary Pancreat Surg* 2004, **11:**301–309.
16. Wang T, Liang YM, Hu P, Cheng YF: **Mucins differently expressed in various ampullary adenocarcinomas.** *Diagn Pathol* 2011, **6:**102.
17. Westgaard A, Tafjord S, Farstad IN, Cvancarova M, Eide TJ, Mathisen O, Clausen OP, Gladhaug IP: **Resectable adenocarcinomas in the pancreatic head: the retroperitoneal resection margin is an independent prognostic factor.** *BMC cancer* 2008, **8:**5.
18. Rau BM, Moritz K, Schuschan S, Alsfasser G, Prall F, Klar E: **R1 resection in pancreatic cancer has significant impact on long-term outcome in standardized pathology modified for routine use.** *Surgery* 2012, **152:**S103–111.

doi:10.1186/1746-1596-9-80
Cite this article as: Elebro and Jirström: Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma. *Diagnostic Pathology* 2014 **9**:80.

Paper II

Elebro et al. Journal of Translational Medicine 2014, 12:289
http://www.translational-medicine.com/content/12/1/289

JOURNAL OF TRANSLATIONAL MEDICINE

RESEARCH Open Access

Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma

Jacob Elebro[1*], Margareta Heby[1], Alexander Gaber[1], Björn Nodin[1], Liv Jonsson[1], Richard Fristedt[1], Mathias Uhlén[2,3], Karin Jirström[1] and Jakob Eberhard[1]

Abstract

Background: Pancreatic cancer and other pancreaticobiliary type periampullary adenocarcinomas have a dismal prognosis even after resection and neoadjuvant chemotherapy. Intestinal type periampullary adenocarcinomas generally have a better prognosis, but little is known on optimal neoadjuvant and adjuvant treatment. New prognostic and treatment predictive biomarkers are needed for improved treatment stratification of patients with both types of periampullary adenocarcinoma. Expression of the Special AT-rich sequence-binding protein 1 (SATB1) has been demonstrated to confer a worse prognosis in several tumour types, whereas its close homologue SATB2 is a proposed diagnostic and favourable prognostic marker for colorectal cancer. The prognostic value of SATB1 and SATB2 expression in periampullary adenocarcinoma has not yet been described.

Methods: Immunohistochemical expression of SATB1 and SATB2 was analysed in tissue microarrays with primary tumours and a subset of paired lymph node metastases from 175 patients operated with pancreaticoduodenectomy for periampullary adenocarcinoma. Kaplan-Meier and Cox regression analysis were applied to explore the impact of SATB1 and SATB2 expression on recurrence free survival (RFS) and overall survival (OS).

Results: Positive expression of SATB1 was denoted in 16/106 primary pancreatobiliary type tumours and 11/65 metastases, and in 15/63 primary intestinal type tumours and 4/26 metastases, respectively. Expression of SATB1 was an independent predictor of a significantly shorter RFS and OS in pancreatobiliary type, but not in intestinal type adenocarcinomas. Moreover, SATB1 expression predicted an improved response to adjuvant chemotherapy in both tumour types. SATB2-expression was seen in 3/107 pancreatobiliary type primary tumours, and in 8/61 intestinal type primary tumours. The small number of cases with positive SATB2 expression did not allow for any firm conclusions on its prognostic value.

Conclusions: These findings demonstrate the potential utility of SATB1 as a prognostic and predictive biomarker for chemotherapy response in both intestinal type and pancreatobiliary type periampullary adenocarcinomas, including pancreatic cancer.

Keywords: Periampullary adenocarcinoma, Pancreatic cancer, Immunohistochemistry, Biomarkers, Prognosis, Treatment prediction

* Correspondence: jacob.elebro@med.lu.se
[1]Department of Clinical Sciences Lund, Oncology and Pathology, Lund University, Skåne University Hospital, 221 85 Lund, Sweden
Full list of author information is available at the end of the article

Background

Periampullary adenocarcinomas encompass tumours originating in or adjacent to the ampulla of Vater; pancreatic cancer, distal bile duct cancer, ampulla of Vater carcinoma and carcinoma of the periampullary duodenum. Pancreatic cancer is the most common type of periampullary adenocarcinoma, but only a minority can be resected with a curative intent, due to either locally advanced growth or distant metastases at presentation. There are two major morphological types of periampullary adenocarcinomas, which have different prognosis and receive different chemotherapy. Pancreatobiliary type (PB-type) adenocarcinomas include pancreatic cancer, distal bile duct cancer, and some of the ampullary carcinomas. They have a dismal prognosis even after resection and adjuvant gemcitabine-based chemotherapy. Intestinal type (I-type) periampullary adenocarcinomas include duodenal carcinoma and some of the ampullary carcinomas. They have a better prognosis but little is known on risk stratification and optimal chemotherapy [1,2]. Hence, new biomarkers are needed to better stratify both PB-type and I-type periampullary adenocarcinomas according to risk and expected response to treatment.

Special AT-rich sequence-binding protein 1 (SATB1) is a genome organizing protein which regulates region-specific epigenetic modifications and expression of a large number of genes, and special AT-rich sequence-binding protein 2 (SATB2) is a close homologue with similar functions [3-5].

SATB1-expression has been demonstrated to confer a more aggressive tumour phenotype and a shorter patient survival in several cancer forms, e.g. breast cancer [3], prostate cancer [6], laryngeal squamous cell carcinoma [7], nasopharyngeal cancer [8], hepatocellular carcinoma [9], rectal cancer [10], cutaneous malignant melanoma [11], epithelial ovarian cancer [12], glioma [13] and gastric cancer [14].

The SATB2 gene is involved in osteoblast differentiation and craniofacial patterning [15,16] and has been demonstrated to be abundantly expressed in normal colorectal mucosa and colorectal adenocarcinomas, but more sparsely in other types of carcinomas [17]. Low or absent SATB2-expression has further been shown to be a marker of malignant behaviour and poor prognosis in colorectal cancer [18,19], whereas high expression correlated to a better response to neoadjuvant chemotherapy in rectal cancer and neoadjuvant/adjuvant chemotherapy in stage III-IV colorectal cancer [20].

The expression and prognostic significance of SATB1 and SATB2 in pancreatic, distal bile duct, ampullary or duodenal adenocarcinomas has not yet been reported. The aim of the present study was therefore to examine the expression, clinicopathological correlates, and prognostic and treatment predictive ability of SATB1 and SATB2 in primary tumours (n = 175) and paired lymph node metastases (n = 105) from a consecutive cohort of patients with periampullary adenocarcinoma, including pancreatic cancer.

Methods

Patients

The study cohort is a previously described retrospective consecutive series of 175 pancreaticoduodenectomy specimens with primary adenocarcinomas surgically treated at the University hospitals of Lund and Malmö, Sweden, from January 1 2001 until December 31 2011 [21]. Data on survival were gathered from the Swedish National Civil Register. Follow-up started at the date of surgery and ended at death, at 5 years after surgery or at December 31 2013, whichever came first. Information on neoadjuvant and adjuvant treatment and recurrence was obtained from patient records.

All haematoxylin & eosin stained slides from all cases were re-evaluated by one pathologist (JEL), blinded to the original report and outcome, with the decision on tumour origin and morphological type being based on several criteria, as previously described [21].

The study has been approved by the Ethics Committee of Lund University (ref nr 445/07).

Tissue microarray construction

Tissue microarrays (TMAs) were constructed using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminister, MD, USA). A standard set of three tissue cores (1 mm) were obtained from each of the 175 primary tumours and from lymph node metastases from 105 of the cases, whereby one to three lymph node metastases were sampled in each case.

Immunohistochemistry and staining evaluation

For immunohistochemical analysis of SATB1 and SATB2 expression, 4 μm TMA-sections were automatically pre-treated using the PT Link system and then stained in an Autostainer Plus (DAKO; Glostrup, Copenhagen, Denmark) with anti-SATB1, clone EPR3895, Epitomics, Burlingame, CA, USA, and anti-SATB2 #AMAb90679 CL0320, Atlas Antibodies AB, Stockholm, Sweden. Expression of SATB1 and SATB2 was denoted as positive when there was nuclear positivity of any intensity in at least 1 percent of cancer cells. Cases denoted as positive in any of the TMA-cores of the primary tumour or a lymph node metastasis were considered positive. Stromal lymphocytes served as a positive control for SATB1 and normal colorectal mucosa as a positive control for SATB2.

Statistical analysis

Chi square test was applied to analyse the relationship between SATB1 expression and clinicopathological

parameters. Two patients with PB-type adenocarcinomas who had received neoadjuvant chemotherapy were excluded from the correlation and survival analyses. Three additional patients were excluded from the survival analyses; two with I-type adenocarcinomas who died within one month from surgery due to complications and one with PB-type adenocarcinoma who emigrated 5 months after surgery.

Kaplan Meier estimates of 5-year overall survival (OS) and recurrence-free survival (RFS) and log rank test were applied to evaluate survival differences in strata according to positive and negative SATB1 and SATB2 expression. Hazard ratios (HR) for death and recurrence within 5 years were calculated by Cox regression proportional hazard's modelling in unadjusted analysis and in a multivariable model adjusted for age, sex, T-stage, N-stage, differentiation grade, lymphatic invasion, vascular invasion, perineural invasion, infiltration in peripancreatic fat, resection margins, tumour origin, and adjuvant chemotherapy. A backward conditional method was used for variable selection in the adjusted model. To estimate the interaction effect between adjuvant treatment and SATB1 expression in order to measure any possible difference in treatment effect based on SATB1 expression, the following interaction variables were constructed; any adjuvant treatment (+/−) × SATB1 (+/−), and gemcitabine-based treatment (+/−) × SATB1 (+/−).

All tests were two sided. P-values <0.05 were considered significant. All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Associations of SATB1 expression with clinicopathological factors and SATB2 expression

Sample immunohistochemical images of SATB1 and SATB2 expression are shown in Figure 1.

In the full cohort of 175 cases there were 110 PB-type and 65 I-type adenocarcinomas. Two patients with PB-type carcinoma who had received neoadjuvant chemotherapy were excluded from the analyses. Among the remaining cases, SATB1 expression could be assessed in 106/108 (98.1%) primary PB-type carcinomas; 16 (15.1%) being denoted as positive and 90 (84.9%) as negative, and in 65/75 (86.7%) metastases; 11(16.9%) being denoted as positive and 54 (83.1%) as negative. Out of the 11 cases with positive SATB1 expression in a metastasis, 6 (54.5%) had positive and 5 (45.5%) had negative SATB1 expression in the corresponding primary tumour. Using a combined variable wherein SATB1 expression of any intensity in >1% cells in the primary tumour and/or metastases was denoted as positive, 21 (19.8%) PB-type cases had positive and 85 (80.2) cases had negative SATB1-expression (Table 1). SATB1 was assessable in 63/65 (96.9%) primary I-type carcinomas; 15 (23.8%) being positive and 48 (76.2%) being negative, and in 26/30 (86.7%) metastases; 4 (15.4%) being positive and 22 (84.6%) being negative. Out of the 4 cases with positive expression in a

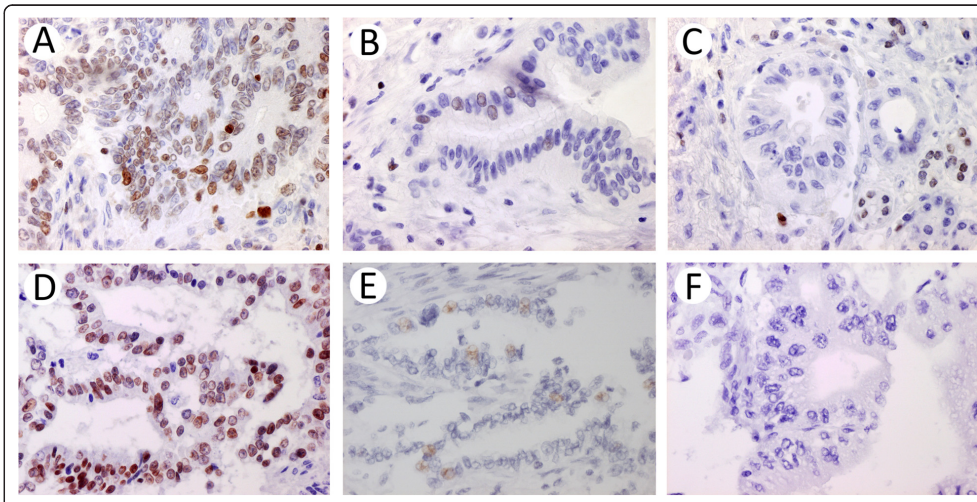

Figure 1 Immunohistochemical stains of SATB1 (A-C) and SATB2 (D-F) showing varying fractions and intensities of positive cells (A-B and D-E) and negative stains (C and F). B and **E** show low fractions of weakly positive cancer cells, in tumours denoted as positive.

Table 1 SATB1-expression in relation to clinicopathological parameters and SATB2-expression

	Pancreatobiliary type				Intestinal type			
	SATB1 - n = 85	SATB1 + n = 21	SATB 1 missing n = 2	p-value	SATB1- n = 47	SATB1+ n = 16	SATB1 missing n = 2	p-value
Age, years, M (IQR)	66 (61–72)	69 (64–74)	2	0.681	67 (62–72)	67 (57–70)	2	0.981
Sex, n (%)				0.469				0.777
Women	41 (48%)	8 (38%)	2		26 (55%)	8 (50%)	1	
Men	44 (52%)	13 (62%)			21 (45%)	8 (50%)	1	
Tumour origin, n (%)				0.852				0.487
Duodenum					12 (26%)	2 (13%)		
Ampulla Intestinal type					35 (74%)	14 (87%)	2	
Ampulla Pancreatobiliary type	16 (19%)	3 (14%)						
Distal bile duct	34 (40%)	10 (48%)	1					
Pancreas	35 (41%)	8 (38%)	1					
Tumour size, mm, M (IQR)	30 (25–35)	28 (21–30)	2	0.799	25 (15–40)	30 (24–40)	2	0.848
Differentiation grade, n (%)				0.211				0.148
Well-moderate	34 (40%)	5 (24%)	1		26 (55%)	5 (31%)	1	
Poor	51 (60%)	16 (76%)	1		21 (45%)	11 (69%)	1	
T-stage, n (%)				1.000				0.860
T1	2 (2%)	0	1		4 (9%)	0	1	
T2	8 (9%)	2 (10%)			8 (17%)	3 (19%)	1	
T3	61 (72%)	16 (76%)	1		18 (38%)	7 (44%)		
T4	14 (16%)	3 (14%)			17 (36%)	6 (37%)		
N-stage, n (%)				0.421				0.564
N0	25 (29%)	4 (19%)	2		26 (55%)	7 (44%)	2	
N1-N2	60 (71%)	17 (81%)			21 (45%)	9 (56%)		
Margins, n (%)				1.000				1.000
R0	5 (6%)	1 (5%)	1		13 (28%)	4 (25%)	1	
R1-Rx	80 (94%)	20 (95%)	1		34 (72%)	12 (75%)	1	
Perineural growth, n (%)				0.232				0.533
No	20 (24%)	2 (10%)	1		34 (72%)	10 (62%)	1	
Yes	65 (76%)	19 (90%)	1		13 (28%)	6 (38%)	1	
Invasion of lymphatic vessels, n (%)				0.792				0.081
No	25 (29%)	7 (33%)	1		25 (53%)	4 (25%)		
Yes	60 (71%)	14 (67%)	1		22 (47%)	12 (75%)	2	
Invasion of blood vessels, n (%)				0.070				0.594
No	60 (71%)	10 (48%)	1		44 (94%)	14 (87%)	2	
Yes	25 (29%)	11 (52%)	1		3 (6%)	2 (13%)		
Growth in peripancreatic fat, n (%)				0.760				0.545
No	18 (21%)	3 (14%)	2		32 (68%)	9 (56%)	2	
Yes	67 (79%)	18 (86%)			15 (32%)	7 (44%)		
SATB2, n (%)				0.092				0.422
Negative	84 (99%)	18 (90%)	2		40 (89%)	13 (81%)	0	
Positive	1 (1%)	2 (10%)	0		5 (11%)	3 (19%)	0	
Missing	0	1	0		2	0	2	

Table 1 SATB1-expression in relation to clinicopathological parameters and SATB2-expression *(Continued)*

Adjuvant chemotherapy, n (%)				0.739			0.301
No adjuvant	41 (48%)	9 (43%)	1	35 (74%)	10 (63%)	2	
5FU-analogue	5 (6%)	3 (14%)		4 (9%)	1 (6%)		
Gemcitabine	35 (41%)	9 (43%)		5 (11%)	2 (13%)		
Gemcitabine + capecitabine	1 (1%)	0	1	0	1 (6%)		
Oxaliplatin +5-FU analogue	1 (1%)	0		3 (6%)	1 (6%)		
Gemcitabine + oxaliplatin	2 (2%)	0		0	1 (6%)		
Recurrence				0.250			0.658
No	16 (19%)	3 (14%)	1	27 (57%)	7 (44%)	1	
Yes, local only	25 (29%)	3 (14%)	1	3 (6%)	1 (6%)		
Yes, non-local	44 (52%)	15 (71%)		17 (36%)	8 (50%)	1	
Included in survival analyses				1.000			1.000
Yes	84 (99%)	21 (100%)	0	45 (96%)	16 (100%)	0	
No	1 (1%)	0	2	2 (4%)	0	2	

There were no significant associations between SATB1-expression, clinicopathological characteristics and SATB2-expression.

metastasis, 3 (75%) also displayed positive expression in the corresponding primary tumour. When combining positivity in primary tumours and/or metastases, there were 16 (25.4%) SATB1 positive and 47 (74.6%) negative I-type cases (Table 1).

There were no significant associations between SATB1-expression and clinicopathological parameters (Table 1). Among SATB1-positive PB-cases there was a tendency towards a higher proportion of cases with blood vessel involvement (p = 0.070), compared with SATB1-negative cases. Among SATB1-positive I-type cases there was a tendency towards a higher proportion of cases with lymphatic vessel involvement (p = 0.081), compared with SATB1-negative cases.

SATB2 expression was assessable in 107/108 (99.1%) PB-type primary tumours, and denoted as positive in 3 (2.8%) cases and negative in 104 (97.2%) cases. There were 2 positive PB-type metastases, both corresponding to positive primary tumours. Among 61/65 (93.8%) assessable I-type primary tumours SATB2 was positive in 8 (13.1%), and negative in 53 (86.9%) cases. There were 3 positive I-type metastases, all corresponding to positive primary tumours.

SATB1 expression was positive in 2 and negative in 1 of the 3 cases with SATB2-positive PB-type tumours. Three of the 8 SATB2-positive I-type cases were SATB1-positive, and 5 were negative. There were no significant associations between SATB1 and SATB2 expression in either of the morphological groups (Table 1).

SATB2 expression was significantly associated with growth in peripancreatic fat in I-type tumours (p = 0.042), but not with any other clinicopathological factor, and there were no significant associations in PB-type tumours (Additional file 1: Table S1).

There was a significant association between gemcitabine based adjuvant chemotherapy and tumour origin in PB-type tumours, and between adjuvant chemotherapy and involved lymph nodes in intestinal type tumours (Table 2). Except for these two factors, the distribution of patient and tumour characteristics did not differ significantly between patients who had received or not received adjuvant chemotherapy in neither of the histological subtypes.

Prognostic and treatment predictive value of SATB1 expression in pancreatobiliary type tumours

As demonstrated in Figure 2A-B, Kaplan-Meier analysis revealed that SATB1 expression was prognostic for OS and RFS in the PB-group of tumours. SATB1 positive cases had a shorter OS compared with SATB1 negative cases, median 16.7 months (interquartile range, IQR 9.9-25.1) vs 27.3 months (IQR 15.8-46.3) (logrank p = 0.004), and also a shorter RFS, median 9.0 months (IQR 5.1-18.8) vs 16.8 months (IQR 8.0-28.5) (logrank p = 0.018). As demonstrated in Table 3, the significant associations of SATB1 expression with survival were confirmed in Cox univariable analysis for both OS (HR = 2.11; 95% confidence interval, CI 1.25-3.56) and RFS (HR = 1.87; 95% CI 1.10-3.18), and this significance was retained for OS in multivariable analysis (HR = 1.79; 95% CI 1.05-3.05).

SATB1-positive cases receiving adjuvant gemcitabine had a prolonged OS, median 24.7 (IQR 18.2-41.1), compared with SATB1-positive cases not receiving adjuvant gemcitabine, median 9.9 (IQR 8.3-14.6) (logrank p = 0.048, Figure 2C), while there was no significant difference in OS between SATB1-negative cases receiving (38/84) or not receiving (46/84) adjuvant gemcitabine (Figure 2C). The

Table 2 Adjuvant chemotherapy in relation to clinicopathological parameters

	Pancreatobiliary type			Intestinal type		
	No adjuvant or non-gemcitabine based n = 60	Gemcitabine based n = 50	p-value	No adjuvant n = 47	Any adjuvant n = 18	P-value
No follow up, n	1	0	1.000	2	0	1.000
Received neoadjuvant treatment, n	0	2	0.204	0	0	
Sex			0.253			1.000
Female, n (%)	31 (61%)	20 (39%)		25 (71%)	10 (29%)	
Male, n (%)	29 (49%)	30 (51%)		22 (73%)	8 (27%)	
Age at surgery, years. M (IQR)	69 (62–73)	66 (60–70)	0.260	67 (62–72)	67 (56–71)	0.441
Tumour origin			**0.002**			0.316
Pancreas, n (%)	16 (35%)	30 (65%)				
Distal bile duct, n (%)	30 (67%)	15 (33%)				
Ampulla of Vater, n (%)	14 (74%)	5 (26%)		35 (69%)	16 (31%)	
Duodenum, n (%)				12 (86%)	2 (14%)	
Tumour size, mm. M (IQR)	30 (22–37)	30 (25–35)	0.702	23 (13–40)	30 (24.5-40)	0.690
Tumour grade			0.555			0.783
Well/moderate, n (%)	21 (50%)	21 (50%)		24 (75%)	8 (25%)	
Poor, n (%)	39 (57%)	29 (43%)		23 (70%)	10 (30%)	
Lymph nodes			0.531			**0.013**
Uninvolved (N0), n (%)	20 (61%)	13 (39%)		30 (86%)	5 (14%)	
Involved (N1-N2), n (%)	40 (52%)	37 (48%)		17 (57%)	13 (43%)	
Margins			0.452			0.230
Uninvolved, n (%)	5 (71%)	2 (29%)		11 (61%)	7 (39%)	
Involved or unknown, n (%)	55 (53%)	48 (47%)		36 (77%)	11 (23%)	
Perineural growth			0.362			0.229
No, n (%)	16 (64%)	9 (36%)		35 (78%)	10 (22%)	
Yes, n (%)	44 (52%)	41 (48%)		12 (60%)	8 (40%)	
Growth in lymph vessels			0.223			1.000
No, n (%)	16 (46%)	19 (54%)		21 (72%)	8 (28%)	
Yes, n (%)	44 (59%)	31 (41%)		26 (72%)	10 (28%)	
Growth in blood vessels			0.312			1.000
No, n (%)	37 (51%)	36 (49%)		43 (72%)	17 (28%)	
Yes, n (%)	23 (62%)	14 (38%)		4 (80%)	1 (20%)	
Growth in peripancreatic fat			0.649			0.142
No, n (%)	15 (60%)	10 (40%)		34 (79%)	9 (21%)	
Yes, n (%)	45 (53%)	40 (47%)		13 (59%)	9 (41%)	
T-stage			0.240			0.301
T1, n (%)	2 (67%)	1 (33%)		5 (100%)	0	
T2, n (%)	4 (33%)	8 (67%)		10 (83%)	2 (17%)	
T3, n (%)	42 (54%)	36 (46%)		18 (72%)	7 (28%)	
T4, n (%)	12 (71%)	5 (29%)		14 (61%)	9 (39%)	
Year of surgery. M (IQR)	2007.5 (2004–2010)	2009 (2007–2010)	**0.004**	2006 (2003–2009)	2009 (2006.5-2010)	0.372

M, median. IQR, interquartile range. Bold text indicates significant p-values.

interaction between SATB1 and adjuvant gemcitabine in relation to OS approached significance, p(interaction) = 0.066 (Table 4).

Similar findings were obtained when considering SATB1 expression in primary tumours only; with a significantly shorter OS for SATB1 positive PB-cases

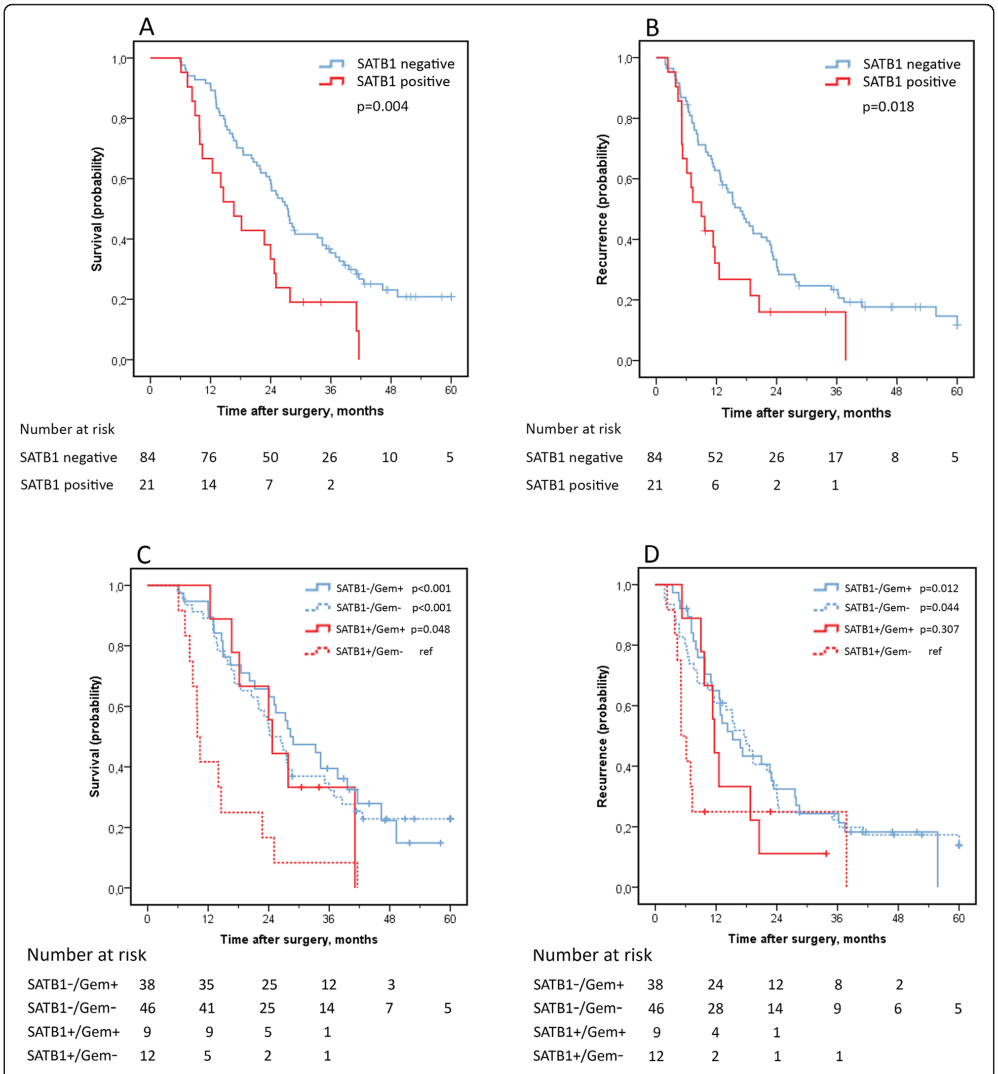

Figure 2 Kaplan-Meier estimates of overall survival (A) and recurrence free survival (B) in pancreatobiliary type tumours stratified by SATB1-expression and corresponding curves stratified for adjuvant chemotherapy (C-D).

(logrank p = 0.021) and a difference in response to adjuvant gemcitabine in SATB1 positive cases (8/16 receiving vs 8/16 not receiving adjuvant gemcitabine, logrank p = 0.054) compared with negative cases (39/89 receiving vs 50/89 not receiving adjuvant gemcitabine, logrank p = 0.491) and p(interaction) =0.067.

Prognostic and treatment predictive value of SATB1 expression in intestinal type tumours

In contrast to the PB-group, SATB1 expression was not prognostic for OS or RFS in the I-type category of tumours (Figure 3A-B). However, while there was no significant difference in OS or RFS between SATB1-negative

Table 3 Hazard ratios for overall survival and recurrence free survival in pancreatobiliary type tumours

	Pancreatobiliary type			
	OS		RFS	
	Univariable	Multivariable	Univariable	Multivariable
Age	0.99 (0.96-1.02)	1.02 (0.99-1.05)	0.98 (0.96-1.01)	0.99 (0.96-1.02)
Sex				
Women				
Men	1.24 (0.80-1.91)	1.02 (0.64-1.64)	1.09 (0.72-1.66)	0.82 (0.52-1.30)
Tumour size				
	1.03 (1.01-1.05)	1.01 (0.99-1.04)	**1.04 (1.02-1.05)**	1.01 (0.99-1.04)
Tumour grade				
Well-moderate				
Poor	**2.50 (1.54-4.05)**	**2.10 (1.28-3.45)**	**2.40 (1.50-3.83)**	**2.35 (1.43-3.84)**
Tumour origin				
Ampulla				
Distal bile duct	0.74 (0.40-1.34)	1.02 (0.53-1.98)	1.10 (0.61-1.97)	2.68 (0.33-21.81)
Pancreas	0.88 (0.49-1.60)	1.08 (0.56-2.08)	1.01 (0.56-1.84)	2.26 (0.27-18.81)
T-stage				
T1				
T2	1.93 (0.23-16,04)	0.54 (0.06-5.05)	2.21 (0.27-18.38)	0.61 (0.06-5.75)
T3	3.99 (0.55-28.85)	0.74 (0.09-6.10)	6.43 (0.89-46.43)	1.28 (0.16-10.29)
T4	5.11 (0.67-38.79)	2.36 (0.11-49.41)	5.95 (0.78-45.43)	0.93 (0.11-8.00)
N-stage				
N0				
N1	**2.55 (1.49-4.38)**	**2.49 (1.42-4.38)**	**2.59 (1.55-4.33)**	**2.15 (1.22-3.80)**
Margin status				
R0				
R1-Rx	4.02 (0.99-16.38)	2.43 (0.59-10.02)	2.71 (0.99-7.44)	2.30 (0.82-6.50)
Perineural				
Pn0				
Pn1	**1.97 (1.10-3.53)**	1.04 (0.50-2.15)	**3.09 (1.66-5.75)**	1.80 (0.94-3.46)
Lymphatic vessels				
L0				
L1	1.57 (0.96-2.56)	1.02 (0.57-1.85)	**1.85 (1.14-3.01)**	1.14 (0.65-2.00)
Blood vessels				
V0				
V1	**2.43 (1.56-3.78)**	**2.53 (1.59-4.03)**	**2.35 (1.50-3.69)**	**1.96 (1.21-3.17)**
Peripancreatic fat				
Pn0				
Pn1	**1.89 (1.05-3.40)**	0.94 (0.47-1.90)	**2.75 (1.50-5.02)**	1.78 (0.94-3.40)
SATB1				
Negative				
Positive	**2.11 (1.25-3.56)**	**1.79 (1.05-3.05)**	**1.87 (1.10-3.18)**	1.54 (0.89-2.66)
Adjuvant chemotherapy				
None/other				
Gemcitabine	0.76 (0.49-1.18)	**0.56 (0.35-0.89)**	0.98 (0.64-1.49)	0.72 (0.46-1.12)

Bold text indicates significant values.

Table 4 Cox proportional hazards analysis of the impact of SATB1 protein expression on overall survival and recurrence free survival in resected pancreatobiliary type and intestinal type periampullary adenocarcinomas

	OS			RFS		
Pancreatobiliary type	**HR (95% CI)**	**n (events)**	**p†**	**HR (95% CI)**	**n (events)**	**p†**
All cases						
SATB1 neg	1.00	84 (63)		1.00	84 (69)	
SATB1 pos	**2.11 (1.25-3.56)**	21 (19)		**1.87 (1.10-3.18)**	21 (18)	
No adjuvant treatment						
SATB1 neg	1.00	40 (30)		1.00	40 (33)	
SATB1 pos	**2.94 (1.37-6.29)**	9 (9)	0.166	1.63 (0.71-3.74)	9 (7)	0.927
Any adjuvant treatment						
SATB1 neg	1.00	44 (33)		1.00	44 (36)	
SATB1 pos	1.70 (0.83-3.52)	12 (10)		**2.05 (1.02-4.11)**	12 (11)	
No gemcitabine						
SATB1 neg	1.00	46 (35)		1.00	46 (38)	
SATB1 pos	**3.14 (1.60-6.16)**	12 (12)	0.066	**2.05 (1.00-4.20)**	12 (10)	0.384
Gemcitabine						
SATB1 neg	1.00	38 (28)		1.00	38 (31)	
SATB1 pos	1.44 (0.62-3.35)	9 (7)		1.60 (0.72-3.56)	9 (8)	
Intestinal type						
All cases						
SATB1 neg	1.00	45 (22)		1.00	45 (20)	
SATB1 pos	1.06 (0.47-2.38)	16 (8)		1.26 (0.57-2.77)	16 (9)	
No adjuvant treatment						
SATB1 neg	1.00	33 (17)		1.00	33 (13)	
SATB1 pos	1.62 (0.67-3.92)	10 (7)	0.165	**2.69 (1.11-6.51)**	10 (8)	**0.021**
Any adjuvant treatment						
SATB1 neg	1.00	12 (5)		1.00	12 (7)	
SATB1 pos	0.30 (0.03-2.56)	6 (1)		0.18 (0.02-1.46)	6 (1)	
No gemcitabine						
SATB1 neg	1.00	40 (20)		1.00	40 (17)	
SATB1 pos	1.20 (0.51-2.83)	12 (7)	0.649	1.76 (0.76-4.09)	12 (8)	0.143
Gemcitabine						
SATB1 neg	1.00	5 (2)		1.00	5 (3)	
SATB1 pos	0.67 (0.06-7.53)	4 (1)		0.27 (0.03-2.64)	4 (1)	

†P value for term of interaction by Cox multivariable analysis including treatment, SATB1 expression, gemcitabine vs no gemcitabine or any adjuvant vs no adjuvant, and a term of interaction. Bold text indicates significant values.

cases receiving (12/45) or not receiving (33/45) adjuvant chemotherapy (logrank p = 0.866), there was a tendency towards a prolonged OS for cases with SATB1-positive tumours receiving adjuvant chemotherapy (6/16), median n.r. (IQR 40.2-n.r.), compared with SATB1-positive cases not receiving adjuvant chemotherapy (10/16), median 29.7 (IQR 20.9-54.3) (logrank p = 0.093) (Figure 3C). SATB1-positive cases receiving adjuvant chemotherapy (6/16) also had a prolonged RFS, median n.r. (IQR n.r-n.r.), compared with SATB1-positive cases not receiving adjuvant chemotherapy (10/16), median 13.6 (IQR 7.2-35.9) (logrank p = 0.022) and there was a tendency towards a prolonged RFS in SATB1-positive cases receiving adjuvant chemotherapy compared to SATB1-negative cases receiving adjuvant chemotherapy (logrank p = 0.071). There was no significant difference in RFS between SATB1-negative cases receiving (12/45) or not receiving adjuvant chemotherapy (33/45) (logrank p = 0.257) (Figure 3D). There was a significant interaction between SATB1 and adjuvant chemotherapy in relation to RFS in I-type tumours, p(interaction) = 0.021.

Similar results were seen when considering SATB1 expression in primary I-type tumours only; no difference in RFS between SATB1-negative cases receiving or not

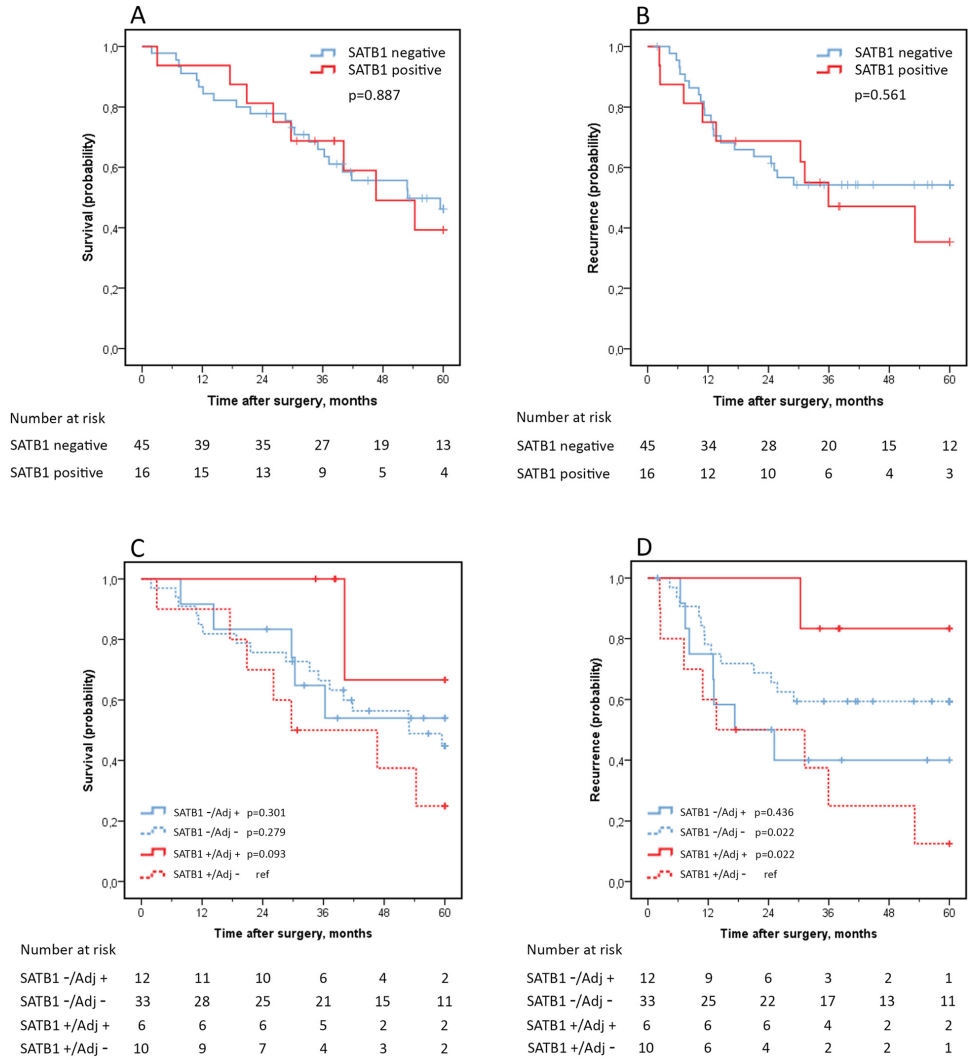

Figure 3 Kaplan-Meier estimates of overall survival (A) and recurrence free survival (B) in intestinal type tumours stratified by SATB1-expression and corresponding curves stratified for adjuvant chemotherapy (C-D).

receiving adjuvant chemotherapy (logrank p = 0.332) while RFS differed significantly between SATB1-positive cases receiving or not receiving adjuvant chemotherapy (logrank p = 0.031). The interaction between SATB1 and adjuvant chemotherapy in relation to RFS was significant also when considering positivity in primary tumours only, p(interaction) = 0.032.

Prognostic and treatment predictive value of SATB2 expression

SATB2-expression was only seen in 3 out of 107 PB-type tumours, making the statistical analyses hazardous to interpret. However, as demonstrated in Figure 4A-B, a significantly shorter OS and RFS was observed for the small number of cases having SATB2-positive tumours, and this

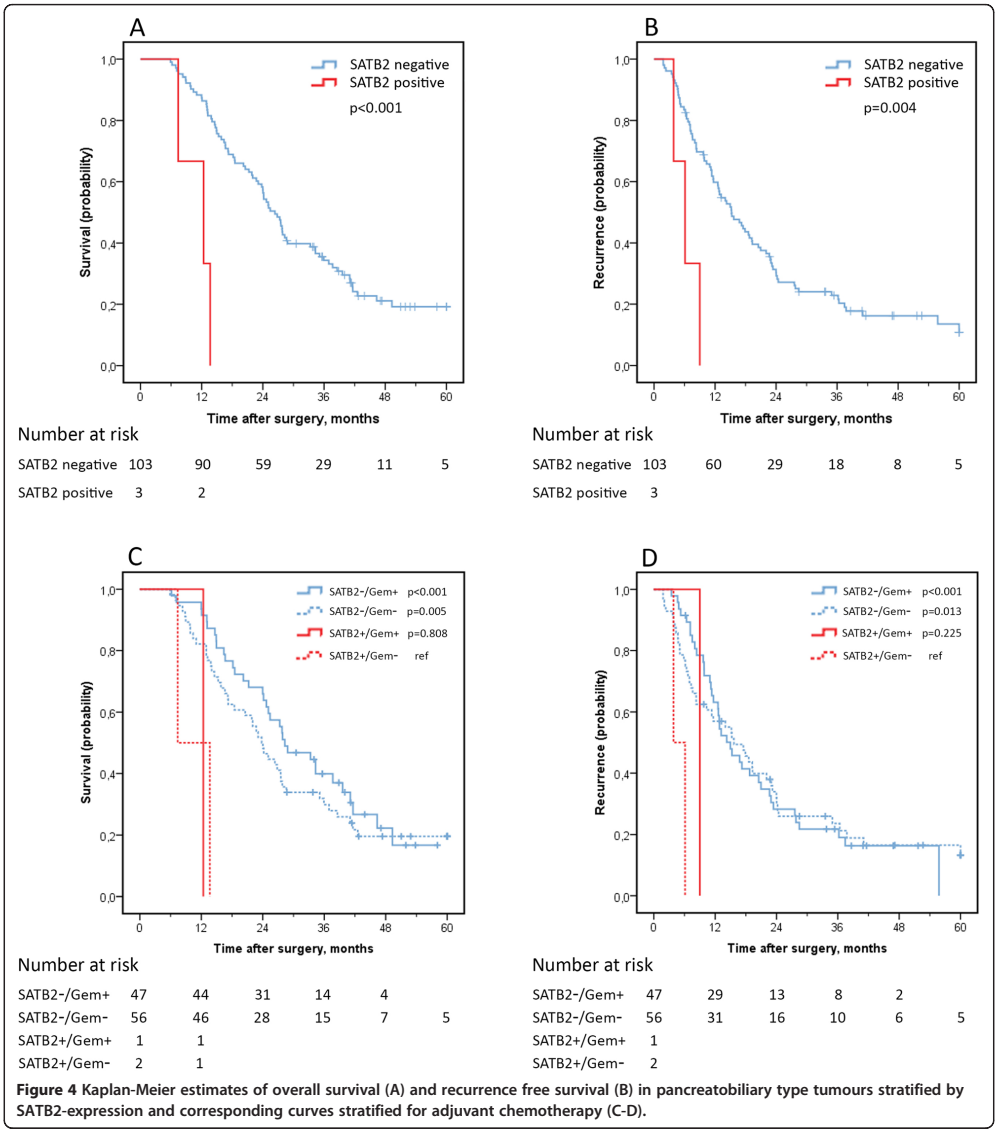

Figure 4 Kaplan-Meier estimates of overall survival (A) and recurrence free survival (B) in pancreatobiliary type tumours stratified by SATB2-expression and corresponding curves stratified for adjuvant chemotherapy (C-D).

significance was retained in both univariable analysis for OS and RFS (HR 7.79; 95% CI 2.29-26.51 and HR 4.93; 95% CI 1.50-16.2) and in multivariable analysis for OS and RFS (HR 4.08; 95% CI 1.18-14.11 and HR 6.40; 95% CI 1.90-21.58).

In I-type tumours, SATB2-positivity was seen in 8 out of 61 cases. Expression of SATB2 was however not prognostic, for OS or RFS (Figure 5A-B). Moreover, there were no significant differences in survival between SATB2-positive cases receiving or not receiving adjuvant chemotherapy, but, of note, there were no recurrences or fatalities among SATB2-positive I-type cases receiving adjuvant chemotherapy (Figure 5C-D).

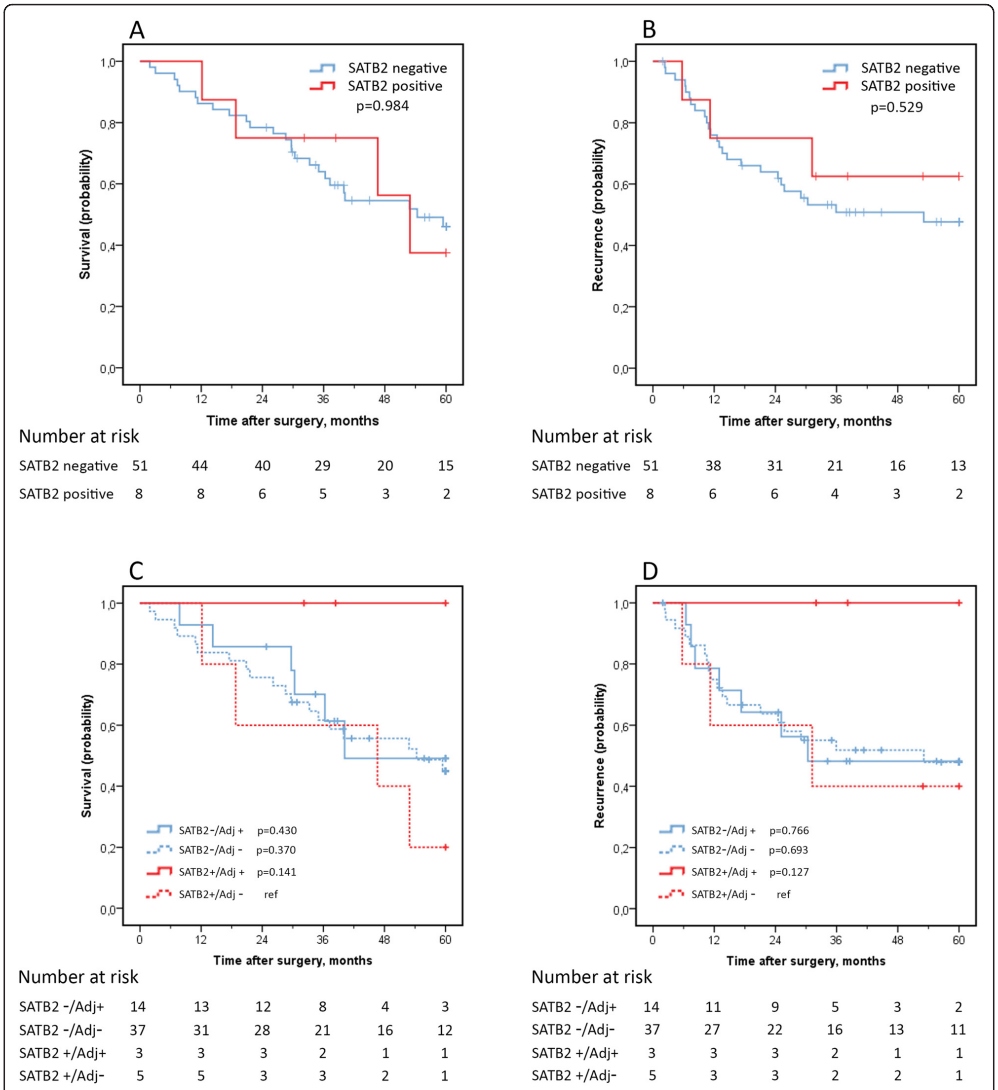

Figure 5 Kaplan-Meier estimates of overall survival (A) and recurrence free survival (B) in intestinal type tumours stratified by SATB2-expression and corresponding curves stratified for adjuvant chemotherapy (C-D).

Discussion

The results from this study provide a first demonstration of the expression and prognostic value of SATB1 in pancreatic, distal bile duct, ampullary and duodenal adenocarcinoma. Positive SATB1-expression was observed in 20% of resected PB-type cases, and was associated with a shorter RFS and OS, which is in line with previous publications on the prognostic significance of SATB1 expression in several other major types of cancer [3,7,9-12,14]. The findings from the present study thus provide further evidence of SATB1 being a master regulator towards a more aggressive tumour phenotype and a biomarker of poor

prognosis in human cancer. In addition, the finding of a potential treatment predictive role of SATB1, its expression being associated with a better response to adjuvant gemcitabine in PB-type tumours, reflected in a prolonged 5-year survival, and an improved response to any adjuvant chemotherapy in I-type tumours, reflected in a prolonged recurrence-free survival, has however not yet been described in any type of cancer. Patients with pancreatic and periampullary adenocarcinomas have a very dismal prognosis even after surgical removal of the tumour. According to contemporary treatment protocols, all patients with pancreatobiliary type adenocarcinoma, including pancreatic cancer are recommended adjuvant treatment, and adjuvant chemotherapy with gemcitabine has recently been shown to increase overall and disease-free survival among patients with radically resected tumours [22]. A challenging task is however to identify which patients will actually benefit from this treatment and not only suffer from the adverse side effects resulting in a reduced quality of life. The here examined retrospective cohort consists of a comparatively large proportion of patients who did not receive any adjuvant chemotherapy, which is in part likely due to the fact that all types of periampullary adenocarcinomas are included. As shown in Table 2, tumour origin and year of surgery differs between the gemcitabine and non-gemcitabine groups of PB-type tumours. During the first part of the included period (2001–2011), the distinction between pancreatobiliary and intestinal tumour morphology was not made, and decision on adjuvant chemotherapy seems to have been based mainly on tumour origin. Many PB-type ampullary tumours did thus not receive adjuvant chemotherapy and tumours of distal bile duct origin were given adjuvant chemotherapy less often than tumours of pancreatic origin. For intestinal type tumours, decision on adjuvant chemotherapy seems to have been based primarily on involved lymph nodes, as this is the only parameter that differs significantly between the group that received and those that did not receive adjuvant chemotherapy. Although treatment predictive effects are best studied in a randomized setting, the nearly equal distribution of patients treated or not treated with adjuvant chemotherapy in this retrospective cohort provides a better setting for discovery of potential treatment predictive markers than studies on cohorts where all patients have received adjuvant chemotherapy.

Apart from considerations in the adjuvant situation, SATB1 could also prove to be a useful biomarker for identification of patients with borderline resectable tumours who will respond well to neoadjuvant chemotherapy, thus increasing the number of resectable tumours. Therefore, the indication of a treatment predictive value of SATB1 expression in periampullary adenocarcinoma is of high potential clinical relevance and merits further validation in additional patient cohorts. The mechanistic basis for SATB1-related increased sensitivity to various combinations of chemotherapy should also be pursued in future studies.

Given the high homology of SATB1 and SATB2, it is important to use well-validated antibodies to ensure target specificity. The antibodies used in the present study have been validated previously [23] and cross-reactivity should therefore not be an issue.

SATB1 was often heterogeneously expressed and the number of positive cells was often low, which justifies assessment not only of the primary tumour, but also metastases in order to improve the detection of positive cases.

Immunohistochemistry has several advantages compared to e.g. analyses of mRNA levels in that it allows for assessment of candidate protein biomarkers in a morphological and subcellular context. The results from this study are quite in line with several previous studies demonstrating that even a small fraction of SATB1 positive cells by immunohistochemistry is sufficient to confer a poor prognosis [3,12]. Moreover, results from studies on the prognostic value of mRNA levels of SATB1 have shown discrepant results in relation to its protein expression in e.g. breast cancer [24,25]. A likely explanation for this is the more or less abundant expression of SATB1 in activated lymphocytes, also serving as a positive internal control in immunohistochemical studies. Therefore, immunohistochemistry should be the method of choice for assessment of the utility of SATB1 as a prognostic and treatment predictive biomarker in human cancer.

Some methodological aspects on the TMA technique need consideration. Although heterogeneity issues cannot be fully circumvented, it is reasonable to assume that analysis of whole tissue sections will lead to an improved detection rate of positive primary tumours and/or metastases. However, while the use of whole sections is feasible in the clinical setting and in prospective studies, the TMA technique has become a well-established platform for high-throughput tissue biomarker studies in the retrospective setting, and has been demonstrated to provide similar or even better prognostic information for heterogeneously expressed markers than whole section-based analyses [26]. Moreover, a comparative strength of the here used TMA is that tissue cores had, whenever possible, been obtained from different donor blocks of the primary tumours, and from different lymph node metastases in cases with more than one metastasis.

In a previous study related to the potential utility of SATB2 as a diagnostic marker for colorectal cancer, screening of its expression in a multitude of normal and cancerous tissues revealed that none out of 25 pancreatic adenocarcinomas and only one out of 15 bile duct adenocarcinomas were positive for SATB2 [17], which is in line with the finding in the present study of SATB2

being positive in only three out of 107 pancreatobiliary type adenocarcinomas. Although a significant association was found between SATB2 expression and poor prognosis, the small number of positive cases makes it hazardous to draw any conclusions on the potential prognostic value of SATB2 expression in PB-type tumours. In the group of I-type tumours, where SATB2 expression was more frequent (8/61), no prognostic effect was seen. Moreover, while there were no significant treatment predictive effects of SATB2 expression in I-type adenocarcinoma, it is noteworthy that SATB2-positive I-type cases receiving adjuvant chemotherapy had no recurrences or fatalities during the follow up period. This observation may suggest a similar treatment predictive function for SATB2 in I-type tumours as observed for SATB1 and would also be in line with the previously described treatment predictive function of SATB2 in colorectal adenocarcinoma [20]. Along this line, while some studies have suggested antagonistic effects of SATB1 and SATB2 [5,18,23], it cannot be ruled out that SATB1 and SATB2 both increase chemotherapy sensitivity in the here examined types of cancer.

Conclusions

Expression of SATB1 is associated with poor prognosis in pancreatobiliary type adenocarcinomas, and predicts response to adjuvant treatment in both intestinal type and pancreatobiliary type periampullary adenocarcinomas, including pancreatic cancer. These findings are of potential clinical relevance and merit further validation in additional patient cohorts as well as in a mechanistic context.

Additional file

Additional file 1: Table S1. SATB2-expression in relation to clinicopathological parameters and SATB1-expression.

Abbreviations

SATB1: Special AT-rich sequence-binding protein 1; SATB2: Special AT-rich sequence-binding protein 2; PB-type: Pancreatobiliary type adenocarcinoma; I-type: Intestinal type adenocarcinoma; TMA: Tissue microarray; OS: Overall survival; RFS: Recurrence free survival; HR: Hazard ratio; CI: Confidence interval; IQR: Interquartile range; N.r.: Not reached.

Competing interest

The authors declare no competing interest.

Authors' contributions

JEL collected clinicopathological data, assisted with TMA construction, evaluated immunohistochemical stainings, performed the statistical analyses and drafted the manuscript. AG evaluated immunohistochemical stainings. BN constructed the tissue micro array and performed the IHC stainings. MU contributed with antibody validation. JEB, MH, LJ and RF collected clinical data. JEB and KJ conceived the study and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This study was supported by grants from the Knut and Alice Wallenberg Foundation, the Swedish Cancer Society, Lund University Faculty of Medicine and University Hospital Research Grants and Governmental Funding of Clinical Research from the National Health Services (ALF).

Author details

[1]Department of Clinical Sciences Lund, Oncology and Pathology, Lund University, Skåne University Hospital, 221 85 Lund, Sweden. [2]Science for Life Laboratory, Royal Institute of Technology, 171 21 Stockholm, Sweden. [3]School of Biotechnology, AlbaNova University Center, Royal Institute of Technology, 106 91 Stockholm, Sweden.

Received: 4 August 2014 Accepted: 5 October 2014

References

1. Romiti A, Barucca V, Zullo A, Sarcina I, Di Rocco R, D'Antonio C, Latorre M, Marchetti P: **Tumors of ampulla of Vater: A case series and review of chemotherapy options.** *World J Gastrointest Oncol* 2012, **4:**60–67.
2. Westgaard A, Pomianowska E, Clausen OP, Gladhaug IP: **Intestinal-type and pancreatobiliary-type adenocarcinomas: how does ampullary carcinoma differ from other periampullary malignancies?** *Ann Surg Oncol* 2013, **20:**430–439.
3. Han HJ, Russo J, Kohwi Y, Kohwi-Shigematsu T: **SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis.** *Nature* 2008, **452:**187–193.
4. Dobreva G, Dambacher J, Grosschedl R: **SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression.** *Genes Dev* 2003, **17:**3048–3061.
5. Kohwi-Shigematsu T, Poterlowicz K, Ordinario E, Han HJ, Botchkarev VA, Kohwi Y: **Genome organizing function of SATB1 in tumor progression.** *Semin Cancer Biol* 2013, **23:**72–79.
6. Mao L, Yang C, Wang J, Li W, Wen R, Chen J, Zheng J: **SATB1 is overexpressed in metastatic prostate cancer and promotes prostate cancer cell growth and invasion.** *J Transl Med* 2013, **11:**111.
7. Zhao XD, Ji WY, Zhang W, He LX, Yang J, Liang HJ, Wang LL: **Overexpression of SATB1 in laryngeal squamous cell carcinoma.** *ORL J Otorhinolaryngol Relat Spec* 2010, **72:**1–5.
8. Shen Z, Zeng Y, Guo J, Wu Y, Jiang X, Ding R, Wu C, Li R, Luo B, Zeng C, Jiang H, Jie W: **Over-expression of the special AT rich sequence binding protein 1 (SATB1) promotes the progression of nasopharyngeal carcinoma: association with EBV LMP-1 expression.** *J Transl Med* 2013, **11:**217.
9. Tu W, Luo M, Wang Z, Yan W, Xia Y, Deng H, He J, Han P, Tian D: **Upregulation of SATB1 promotes tumor growth and metastasis in liver cancer.** *Liver Int* 2012, **32:**1064–1078.
10. Meng WJ, Yan H, Zhou B, Zhang W, Kong XH, Wang R, Zhan L, Li Y, Zhou ZG, Sun XF: **Correlation of SATB1 overexpression with the progression of human rectal cancer.** *Int J Colorectal Dis* 2012, **27:**143–150.
11. Chen H, Takahara M, Oba J, Xie L, Chiba T, Takeuchi S, Tu Y, Nakahara T, Uchi H, Moroi Y, Furue M: **Clinicopathologic and prognostic significance of SATB1 in cutaneous malignant melanoma.** *J Dermatol Sci* 2011, **64:**39–44.
12. Nodin B, Hedner C, Uhlen M, Jirstrom K: **Expression of the global regulator SATB1 is an independent factor of poor prognosis in high grade epithelial ovarian cancer.** *J Ovarian Res* 2012, **5:**24.
13. Chu SH, Ma YB, Feng DF, Zhang H, Zhu ZA, Li ZQ, Jiang PC: **Upregulation of SATB1 is associated with the development and progression of glioma.** *J Transl Med* 2012, **10:**149.
14. Lu X, Cheng C, Zhu S, Yang Y, Zheng L, Wang G, Shu X, Wu K, Liu K, Tong Q: **SATB1 is an independent prognostic marker for gastric cancer in a Chinese population.** *Oncol Rep* 2010, **24:**981–987.
15. FitzPatrick DR, Carr IM, McLaren L, Leek JP, Wightman P, Williamson K, Gautier P, McGill N, Hayward C, Firth H, Markham AF, Fantes JA, Bonthron DT: **Identification of SATB2 as the cleft palate gene on 2q32-q33.** *Hum Mol Genet* 2003, **12:**2491–2501.
16. Dobreva G, Chahrour M, Dautzenberg M, Chirivella L, Kanzler B, Farinas I, Karsenty G, Grosschedl R: **SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation.** *Cell* 2006, **125:**971–986.
17. Magnusson K, de Wit M, Brennan DJ, Johnson LB, McGee SF, Lundberg E, Naicker K, Klinger R, Kampf C, Asplund A, Wester K, Gry M, Bjartell A, Gallagher WM, Rexhepaj E, Kilpinen S, Kallioniemi OP, Belt E, Goos J, Meijer G, Birgisson H, Glimelius B, Borrebaeck CA, Navani S, Uhlén M, O'Connor DP, Jirström K, Pontén F: **SATB2 in combination with cytokeratin 20 identifies over 95% of all colorectal carcinomas.** *Am J Surg Pathol* 2011, **35:**937–948.
18. Wang S, Zhou J, Wang XY, Hao JM, Chen JZ, Zhang XM, Jin H, Liu L, Zhang YF, Liu J, Ding YQ, Li JM: **Down-regulated expression of SATB2 is associated with metastasis and poor prognosis in colorectal cancer.** *J Pathol* 2009, **219:**114–122.

19. Yang MH, Yu J, Jiang DM, Li WL, Wang S, Ding YQ: **microRNA-182 targets special AT-rich sequence-binding protein 2 to promote colorectal cancer proliferation and metastasis.** *J Transl Med* 2014, **12:**109.
20. Eberhard J, Gaber A, Wangefjord S, Nodin B, Uhlen M, Ericson Lindquist K, Jirstrom K: **A cohort study of the prognostic and treatment predictive value of SATB2 expression in colorectal cancer.** *Br J Cancer* 2012, **106:**931–938.
21. Elebro J, Jirstrom K: **Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma.** *Diagn Pathol* 2014, **9:**80.
22. Oettle H, Neuhaus P, Hochhaus A, Hartmann JT, Gellert K, Ridwelski K, Niedergethmann M, Zulke C, Fahlke J, Arning MB, Sinn M, Hinke A, Riess H: **Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial.** *JAMA* 2013, **310:**1473–1481.
23. Nodin B, Johannesson H, Wangefjord S, DP OC, Ericson-Lindquist K, Uhlen M, Jirstrom K, Eberhard J: **Molecular correlates and prognostic significance of SATB1 expression in colorectal cancer.** *Diagn Pathol* 2012, **7:**115.
24. Hanker LC, Karn T, Mavrova-Risteska L, Ruckhaberle E, Gaetje R, Holtrich U, Kaufmann M, Rody A, Wiegratz I: **SATB1 gene expression and breast cancer prognosis.** *Breast* 2011, **20:**309–313.
25. Iorns E, Hnatyszyn HJ, Seo P, Clarke J, Ward T, Lippman M: **The role of SATB1 in breast cancer pathogenesis.** *J Natl Cancer Inst* 2010, **102:**1284–1296.
26. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, Mross F, Dieterich H, Moch H, Mihatsch M, Kallioniemi OP, Sauter: **Tissue microarrays for rapid linking of molecular changes to clinical endpoints.** *Am J Pathology* 2001, **159:**2249–2256.

doi:10.1186/s12967-014-0289-8
Cite this article as: Elebro *et al.*: Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. *Journal of Translational Medicine* 2014 **12**:289.

Table S1. SATB2-expression in relation to clinicopathological parameters and SATB1-expression.

	Pancreatobiliary type				Intestinal type			
	SATB2- n=104	SATB2+ n=3	SATB2 Missing n=1	p-value	SATB2- n=53	SATB2+ n=8	SATB2 Missing n=4	p-value
Age, years, M (IQR)	67 (62-73)	69	1	0.848	66 (59-71)	68 (61-69)	4	0.950
Sex, n (%)				0.246				0.283
Women	50 (48%)	0	1		28 (53%)	6 (75%)	1	
Men	54 (52%)	3 (100%)	0		25 (47%)	2 (25%)	3	
Tumour origin, n (%)				0.580				0.668
Duodenum					13 (25%)	1 (13%)	0	
Ampulla I- type					40 (75%)	7 (87%)	4	
Ampulla PB- type	18 (17%)	1 (33%)	0					
Distal bile duct	44 (42%)	1 (33%)	0					
Pancreas	42 (41%)	1 (33%)	1					
Tumour size, mm, M (IQR)	30 (23-35)	25	1	0.437	30 (18-40)	18 (11-48)	4	0.363
Differentiation grade, n (%)				1.000				0.260
Well-moderate	39 (38%)	1 (33%)	0		27 (51%)	2 (25%)	3	
Poor	65 (62%)	2 (67%)	1		26 (49%)	6 (75%)	1	
T-stage, n (%)				0.631				0.177
T1	3 (3%)	0	0		2 (4%)	1 (12.5%)	2	
T2	10 (10%)	0	0		10 (19%)	1 (12.5%)	1	
T3	75 (72%)	2 (67%)	1		19 (36%)	5 (62.5%)	1	
T4	16 (15%)	1 (33%)	0		22 (41%)	1 (12.5%)	0	
N-stage, n (%)				1.000				1.000
N0	30 (29%)	1 (33%)	0		28 (53%)	4 (50%)	3	
N1-N2	74 (71%)	2 (67%)	1		25 (47%)	4 (50%)	1	
Margins, n (%)				1.000				1.000
R0	7 (7%)	0	0		15 (28%)	2 (25%)	1	
R1-Rx	97 (93%)	3 (100%)	1		38 (72%)	6 (75%)	3	
Perineural growth, n (%)				0.520				1.000
No	22 (21%)	1 (33%)	0		36 (68%)	6 (75%)	3	
Yes	82 (79%)	2 (67%)	1		17 (32%)	2 (25%)	1	
Invasion of lymphatic vessels, n (%)				1.000				0.710
No	32 (31%)	1 (33%)	0		26 (49%)	3 (38%)	0	
Yes	72 (69%)	2 (67%)	1		27 (51%)	5 (62%)	4	
Invasion of blood vessels, n (%)				0.261				1.000
No	70 (67%)	1 (33%)	0		48 (91%)	8 (100%)	4	
Yes	34 (33%)	2 (67%)	1		5 (9%)	0	0	
Growth in peri-pancreatic fat, n (%)				1.000				**0.042**
No	23 (22%)	0	0		31 (58%)	8 (100%)	4	

Yes	81 (78%)	3 (100%)	1		22 (42%)	0	0	
SATB1, n (%)				0.092				0.422
Negative	84 (81%)	1 (33%)	0		40 (75%)	5 (62%)	2	
Positive	18 (17%)	2 (67%)	1		13 (25%)	3 (38%)	0	
Missing	2 (2%)	0	0		0	0	2	
Adjuvant chemotherapy, n (%)				1.000				0.319
No adjuvant	48 (46%)	2 (67%)	1		39 (74%)	5 (62%)	3	
5FU-analogue	8 (8%)	0	0		5 (9%)	0	0	
Gemcitabine	43 (41%)	1 (33%)	0		5 (9%)	1 (13%)	1	
Gemcitabine + capecitabine	2 (2%)	0	0		1 (2%)	0	0	
Oxaliplatin + 5-FU analogue	1 (1%)	0	0		2 (4%)	2 (25%)	0	
Gemcitabine + oxaliplatin	2 (2%)	0	0		1 (2%)	0	0	
Recurrence				0.422				0.472
No	20 (19%)	0	0		28 (53%)	5 (62%)	2	
Yes, local only	29 (28%)	0	0		3 (5%)	1 (13%)	0	
Yes, non-local	55 (53%)	3 (100%)	1		22 (42%)	2 (25%)	2	
Included in survival analyses				1.000				1.000
Yes	103 (99%)	3 (100%)	1		51 (96%)	8 (100%)	4	
No	1 (1%)	0	0		2 (4%)	0	0	

M, median. IQR, interquartile range. Bold text indicates significant p-values.

Paper III

ACTA ONCOLOGICA
2015; EARLY ONLINE: 1–11
http://dx.doi.org/10.3109/0284186X.2015.1075663

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ORIGINAL ARTICLE

Prognostic effect of hENT1, dCK and HuR expression by morphological type in periampullary adenocarcinoma, including pancreatic cancer

JACOB ELEBRO, LIV BEN DROR, MARGARETA HEBY, BJÖRN NODIN, KARIN JIRSTRÖM & JAKOB EBERHARD

Department of Clinical Sciences, Division of Oncology and Pathology, Lund University, Skåne University Hospital, Lund, Sweden

ABSTRACT

Background: Putative biomarkers of gemcitabine response have been extensively studied in pancreatic cancer, but less so in other types of periampullary adenocarcinoma. The most studied biomarker is human equilibrative nucleoside transporter 1 (hENT1), and the activating enzyme deoxycytidine kinase (dCK) has also been linked to treatment response. The RNA-binding protein human antigen R (HuR) has been demonstrated to confer increased dCK levels in vitro and to predict gemcitabine response in vivo. Here, we investigated the prognostic impact of hENT1, dCK and HuR in pancreatobiliary (PB) and intestinal (I) type periampullary cancers, respectively. *Material and methods*: Immunohistochemical expression of hENT1, dCK and HuR was evaluated in tissue microarrays with all primary tumours and 103 paired lymph node metastases from a consecutive retrospective cohort of 175 patients with resected periampullary adenocarcinomas. *Results*: In patients with PB-type tumours, neither hENT1 nor dCK expression was prognostic. A high HuR cytoplasmic/nuclear ratio was associated with a significantly reduced five-year overall survival (OS) in patients receiving adjuvant gemcitabine (HR 2.07, 95% CI 1.03–4.17) but not in untreated patients ($p_{interaction} = 0.028$). In patients with I-type tumours receiving adjuvant chemotherapy, high dCK expression was significantly associated with a prolonged recurrence-free survival (RFS) (HR 0.09, 95% CI 0.01–0.73, $p_{interaction} = 0.023$). Furthermore, HuR expression was associated with a prolonged OS and RFS in unadjusted but not in adjusted analysis and hENT1 expression was an independent predictor of a prolonged RFS (HR 0.24, 95% CI 0.10–0.59), regardless of adjuvant treatment. *Conclusion*: hENT1 expression is a favourable prognostic factor in I-type, but not in PB-type tumours. High dCK expression is a favourable prognostic factor in patients with I-type tumours receiving adjuvant treatment and a high cytoplasmic/nuclear HuR ratio is a negative prognostic factor in gemcitabine-treated PB-type tumours. Morphological subtype should always be considered in biomarker studies on periampullary cancer.

HISTORY
Received 10 March 2015
Revised 15 July 2015
Accepted 16 July 2015
Published online
9 September 2015

Gemcitabine is an antimetabolite commonly used for treatment of pancreatic cancer and other periampullary adenocarcinomas, in the adjuvant as well as palliative setting. Several putative biomarkers predictive of gemcitabine response have been examined, with varying and sometimes conflicting results.

Human equilibrative nucleoside transporter 1 (hENT1) provides the major route for gemcitabine to enter a cell, and is one of the most extensively studied biomarkers in the context of gemcitabine response. In a meta-analysis encompassing 10 studies on 399 patients with resected pancreatic cancer, hENT1 expression was found to be predictive of gemcitabine response [1]. Results from a retrospective study on 413 consecutive, unselected cases of resected pancreatic cancer showed that hENT1 had no prognostic value in patients receiving non-gemcitabine-based adjuvant therapy, whereas high hENT1 predicted a longer overall survival (OS) among patients who had received gemcitabine [2]. In a study on 196 pancreatic cancer cases from the prospective RTOG 9704 trial, high hENT1 expression was found to correlate with an increased OS and disease-free survival in patients treated with gemcitabine but not with 5-FU [3]. Another study on 380 pancreatic cancer cases from the ESPAC-3 trial demonstrated that resected cases with high tumour-specific hENT1 expression receiving adjuvant gemcitabine had a significantly longer OS than those with low expression, using the median hENT1 score as cut off, but also that patients with low hENT1 had a longer OS after 5-FU therapy than after gemcitabine [4]. The majority of studies have been performed on pancreatic cancer, but in a study on patients with resected ampullary adenocarcinomas, who did not receive adjuvant chemotherapy (n=41), hENT1 expression was found to be higher in intestinal type (I-type) than in pancreatobiliary type (PB-type) tumours [5] and to be associated with a shorter OS [6].

In a first, rate-limiting step, gemcitabine is phosphorylated by deoxycytidine kinase (dCK), which is required for its incorporation into DNA and subsequent masked chain termination and apoptosis [7]. Expression of dCK is required for gemcitabine sensitivity and cell lines with induced resistance show decreased dCK RNA levels, while influx of gemcitabine

Correspondence: J. Elebro, Department of Clinical Sciences, Division of Oncology and Pathology, Lund University, Skåne University Hospital, 221 85 Lund, Sweden. Tel: +46 46 177501, Fax: +46 46 147327. jacob.elebro@med.lu.se

into the cells is unaffected [8]. In a cohort of 416 patients with resected pancreatic cancer, high dCK expression correlated with a significantly longer OS in patients treated with gemcitabine, but not in patients receiving no adjuvant treatment or non-gemcitabine-based adjuvant chemotherapy [2]. In patients with resected pancreatic cancer (n=165) who received adjuvant 5-FU chemoradiation followed by either 5-FU or gemcitabine, high dCK expression correlated with a longer OS in the 5-FU arm but not in the gemcitabine arm [9]. The loss of a treatment predictive effect of dCK in the gemcitabine arm was proposed to be an effect of radiation disrupting the complex of human antigen R (HuR) and dCK-mRNA, leading to lower levels of dCK protein. This hypothesis does however not explain the observed association between high dCK and longer survival in the 5-FU arm. In a meta-analysis including four studies of either protein or gene expression of dCK, high dCK levels predicted a longer OS and recurrence-free survival (RFS) in gemcitabine-treated patients with pancreatic cancer [1]. To our knowledge, the prognostic or predictive value of dCK has not yet been studied in I-type periampullary adenocarcinoma.

HuR is an RNA binding protein that performs post-transcriptional regulation of several proteins in response to stress or growth signals, thereby stabilising mRNAs related to proliferation, angiogenesis and evasion of apoptosis [10,11]. Cytoplasmic HuR (referred to as HuR) is also increased in malignant cells as compared with corresponding normal cells, and has been found to be associated with adverse clinicopathological factors and a shorter OS in several different cancer forms [12], e.g. gastric cancer, gallbladder cancer, breast cancer, urothelial cancer and non-small cell lung cancer. In pancreatic cancer, however, two small studies found high HuR expression to be associated with a longer OS in patients treated with gemcitabine, and HuR was also demonstrated to bind dCK-mRNA, which might explain a greater sensitivity to gemcitabine in tumours with high levels of HuR [13,14]. Low nuclear HuR expression has not been associated with prognosis or prediction of response to chemotherapy, but a high cytoplasmic to nuclear ratio of HuR (HuR C/N ratio) was demonstrated to be associated with a shorter OS in 560 cases of colorectal cancer [15]. The expression of HuR has, to the best of our knowledge, not been studied in I-type periampullary adenocarcinoma before.

Overall, mechanisms and markers of sensitivity to chemotherapy in I-type periampullary adenocarcinomas remain less studied. Therefore, the aim of the present study was to examine the associations between protein levels of hENT1, dCK and HuR, and their prognostic and potential treatment predictive values, in both PB-type periampullary adenocarcinomas, and in I-type periampullary adenocarcinomas.

Patients

The study cohort is a previously described retrospective consecutive series of pancreaticoduodenectomy specimens from all patients ($n = 175$) with periampullary adenocarcinoma, including pancreatic cancer, resected at the university hospitals of Lund and Malmö, Sweden, from 1 January 2001 until 31 December 2011 [16–19]. Data on survival were gathered from the Swedish National Civil Register. Follow-up started at the date of surgery and ended at death, at five years after surgery or at 31 December 2013, whichever came first. Information on neoadjuvant and adjuvant treatment and recurrence was obtained from patient records. All haematoxylin and eosin stained slides from all cases were re-evaluated by one pathologist (JEL), blinded to the original report and outcome, as previously described [16].

The study has been approved by the Ethics Committee of Lund University (ref no 445/07).

Tissue microarray construction

Tissue microarrays (TMAs) were constructed using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminister, MD, USA). A standard set of three tissue cores (1 mm) were obtained from each of the 175 formalin-fixated paraffin-embedded primary tumours and from lymph node metastases from 105 of the cases, whereby one to three lymph node metastases were sampled in each case.

Immunohistochemistry and staining evaluation

For immunohistochemical analysis of dCK and HuR expression, 4 μm TMA-sections were automatically pre-treated using the PT Link system and then stained in an Autostainer Plus (DAKO, Glostrup, Copenhagen, Denmark) with the mouse monoclonal dCK antibody 16G6 (OriGene Technologies, Inc., Rockville, MD, USA) and the mouse monoclonal HuR (G-8) antibody sc-365816 (Santa Cruz Biotechnology Inc., Dallas, TX, USA). For immunohistochemical analysis of HENT1, 4 μm TMA-sections were pre-treated using Cell Condition Solution 1 (Ventana Medical Systems, Tucson, AZ, USA) and stained with the ready-to-use rabbit monoclonal HENT1 antibody SP120 on a Ventana BenchMark stainer (Ventana Medical Systems Inc.).

The staining of dCK and hENT1 was annotated by one pathologist (JEL) and HuR was independently annotated by two observers (JEL and LBD) and consensus was reached in discordant cases. For dCK, only the nuclear staining was scored, HuR and nuclear HuR staining was assessed separately, and for hENT1, cytoplasmic and membranous staining was assessed together. A multiplier of the fraction of stained cells for each level of staining intensity (0=negative, 1=weak, 2=moderate and 3=strong) was calculated for each core (H-score, 0–300) and the mean value of assessable cores was used for further analysis. HuR however often showed varying intensities of weak staining, making it necessary to fine tune the scoring of intensity (0=negative, 1=very weak, 2=weak, 3=weak moderate, 4=strong moderate, 5=strong and 6= very strong), creating a score ranging from 0 to 600. The HuR C/N ratio was calculated using the formula HuR C/N ratio=HuR+0.1/HuRn+0.1, to make cases with no staining computable.

Lymphocytes served as a positive internal control for dCK, endocrine pancreatic islets for HuR, and endocrine pancreatic islets and endothelial cells for hENT1. The median scores of hENT1, dCK, HuR and HuR C/N ratio were calculated separately

for PB- and I-type adenocarcinomas, and were used as cut-offs to create groups of high and low expression.

Statistical analysis

χ^2-test was applied to analyse the relationship between the dichotomised expression of each biomarker and clinicopathological parameters. Two patients with PB-type adenocarcinomas who had received neoadjuvant chemotherapy were excluded from the correlation and survival analyses. Three additional patients were excluded from the survival analyses; two with I-type adenocarcinomas who died within one month from surgery due to complications and one with PB-type adenocarcinoma who emigrated five months after surgery.

Kaplan Meier estimates of five-year OS and RFS and log rank test were applied to evaluate survival differences in strata according to high and low expression for each biomarker combined with given adjuvant treatment; gemcitabine versus none/other for PB-type and any versus none for I-type tumours. Hazard ratios (HR) for death and recurrence within five years were calculated by Cox regression proportional hazard's modelling in unadjusted analysis and in a multivariable model adjusted for expression of hENT1, dCK, HuR and HuR C/N ratio as well as age, T-stage, N-stage, differentiation grade, lymphatic invasion, vascular invasion, perineural invasion, and adjuvant chemotherapy. A backward conditional method was used for variable selection in the adjusted model. To estimate the interaction effect for survival between given adjuvant treatment and the biomarker expression, the following interaction variables were constructed; any adjuvant chemotherapy (+/−) × biomarker (high/low) for I-type, and gemcitabine-based adjuvant treatment (+/−) × biomarker (high/low) for PB-type tumours.

All tests were two sided. p-Values <0.05 were considered significant. All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

The proportional hazards assumption was tested by examining log-log survival curves.

In the planning and execution of this study, efforts were made to follow the REMARK-criteria to increase comparability between studies and enable results to be reproduced [20].

Results

Patient population

In the group of 109 patients with PB-type tumours, 50 received gemcitabine-based adjuvant therapy (45 gemcitabine, 3 gemcitabine + 5-FU analogue and 2 gemcitabine + oxaliplatin) and 59 did not receive adjuvant gemcitabine (50 no adjuvant, 8 5-FU and 1 5-FU + oxaliplatin). Among the 63 patients with I-type tumours, 18 received adjuvant therapy (7 gemcitabine, 5 5-FU, 4 5-FU + oxaliplatin, 1 gemcitabine + 5-FU analogue and 1 gemcitabine + oxaliplatin) and 45 received no adjuvant chemotherapy.

Seven patients with PB-type tumours received adjuvant radiotherapy, six together with 5-FU and one together with gemcitabine. Two patients with I-type tumours received adjuvant radiotherapy, one together with 5-FU and one without chemotherapy.

Median follow up time, from surgery to death, censoring or at the most 60 months, was 25.4 months for PB-type and 38.8 months for I-type tumours.

Median OS for 109 patients with PB-type tumours (84 events) was 25.4 months [95% confidence interval (CI) 22.2–28.7]; 28.1 months (95% CI 26.3–29.9) for 50 patients (37 events) receiving gemcitabine-based adjuvant therapy and 23.1 months (95% CI 19.2–27.0) for 59 patients (47 events) not receiving adjuvant gemcitabine.

Median OS for 63 patients with I-type tumours (32 events) was 52.9 months (95% CI 34.0–71.9); 46.6 months (95% CI 28.9–64.4) for 45 patients not receiving adjuvant treatment, and median OS was not reached for 18 patients who received adjuvant chemotherapy.

hENT1, dCK and HuR expression

Sample immunohistochemical images of hENT1, dCK and HuR stainings are shown in Figure 1. H-score expression levels in PB-type and I-type primary tumours and metastases are shown in Supplementary Figure 1 (available online at http://www.informahealthcare.com).

Independent samples t-test showed a higher expression of hENT1 and HuR, and also a higher HuR C/N ratio in I-type as compared with PB-type primary tumours (all three comparisons $p<0.001$) while there was no difference in expression of dCK by morphological type ($p=0.725$). In PB-type tumours paired samples t-test showed an increased expression of dCK and hENT1 in metastases, as compared with corresponding primary tumours, while in I-type tumours there was a decreased expression of HuR in metastases (Supplementary Figure 1). The HuR C/N ratio did not differ between primary tumours and paired metastases in either morphological type (data not shown).

Paired samples t-test in the full cohort showed an increased dCK H-score from primary tumours to metastases ($p=0.003$) and a decreased HuR H-score ($p=0.002$) while there were no differences in HuR C/N ratio or H-score of hENT1 between primary tumours and metastases (data not shown).

Expression levels of hENT1, dCK and HuR did not differ according to adjuvant treatment (data not shown).

Associations of hENT1, dCK and HuR expression with clinicopathological parameters

Associations between the dichotomised expression of hENT1, dCK and HuR and clinicopathological parameters are shown in Table I for PB-type and in Table II for I-type adenocarcinomas.

In PB-type tumours, dichotomised dCK expression was not significantly associated with other parameters. There were no associations between the dichotomised or continuous H-score of dCK, HuR or HuR C/N ratio in the full cohort or when excluding the nine patients who received adjuvant radiotherapy. HuR was significantly associated with male sex, and hENT1 with well/moderately differentiated PB-type tumours (Table I). As demonstrated in Supplementary Table I (available online at

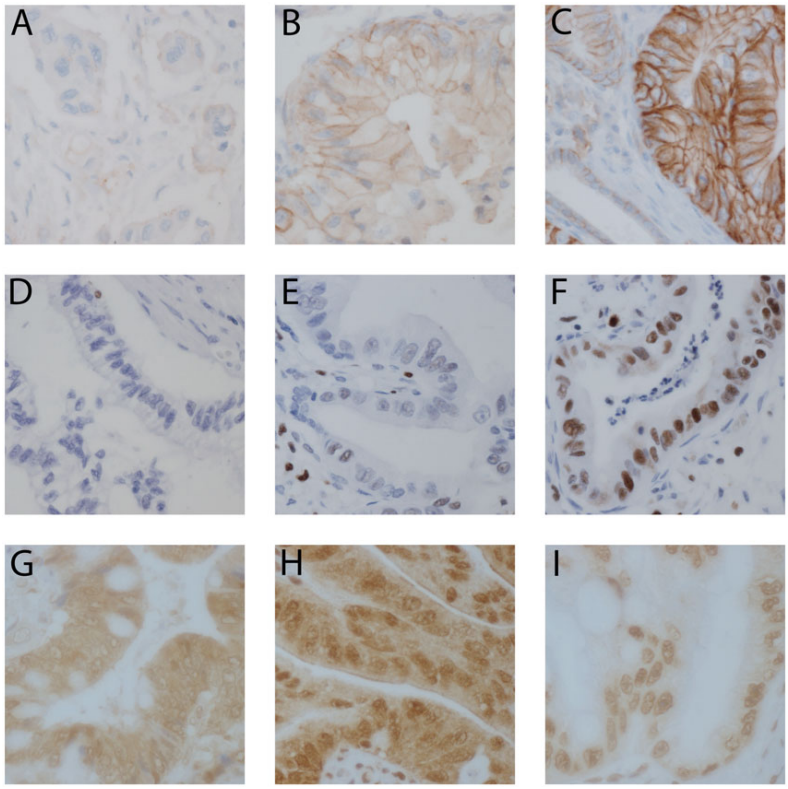


Figure 1. Examples of immunohistochemical staining of hENT1 (A–C), dCK (D–F) and HuR (G–I).

http://www.informahealthcare.com), a high HuR C/N ratio was associated with male sex, high HuR expression and positive or unassessable margins (R1-Rx vs. R0).

In I-type tumours dCK expression was significantly associated with a higher proportion of uninvolved margins, while HuR was associated with hENT1 expression and a lower proportion of perineural growth, and hENT1 was associated with duodenal origin, larger tumour size and uninvolved lymphatic vessels (Table II). There were no significant associations between HuR C/N ratio and any clinicopathological parameter apart from HuR in I-type tumours (data not shown).

Prognostic value of hENT1, dCK and HuR expression

Kaplan–Meier analysis revealed that in the entire group of patients with PB-type tumours, including both those receiving and not receiving adjuvant gemcitabine, there were no differences in OS or RFS according to high or low hENT1, dCK, HuR and HuR C/N ratio (Figure 2A–F, and Supplementary Figure 2, available online at http://www.informahealthcare.com). These findings were confirmed in univariable and multivariable Cox regression analysis for RFS (Table III) and five-year OS (Supplementary Table II, available online at http://www.informahealthcare.com).

Kaplan–Meier analysis revealed that in the entire group of patients with I-type tumours, high hENT1 expression was significantly associated with a longer RFS but not OS, with similar findings in patients not receiving adjuvant therapy (Figure 3A and B). These findings were confirmed in univariable analysis for RFS (HR 0.33, 95% CI 0.15–0.72), and remained significant in multivariable analysis (HR 0.24, 95% CI 0.10–0.59) (Table IV). High hENT1 also had a similar, but borderline significant, prognostic effect for RFS when considering only I-type tumours of ampullary origin, and thus excluding tumours of duodenal origin (data not shown).

In patients with I-type tumours, there was no significant difference in OS or RFS according to high and low dCK expression, neither in the entire group nor in untreated patients (Figure 3C and D). These findings were confirmed in univariable and multivariable Cox regression analysis for RFS

Table I. Associations between hENT1, dCK, HuR and clinicopathological parameters in pancreatobiliary type periampullary adenocarcinomas.

	hENT1			dCK			HuR		
	Low ($n=55$)	High ($n=54$)	p-value	Low ($n=54$)	High ($n=55$)	p-value	Low ($n=55$)	High ($n=54$)	p-value
Excluded, neoadjuvant treatment	1	1			2		1	1	
Lost to follow-up	1			1			1		
hENT1						0.336			0.564
Low				30 (56%)	24 (44%)		29 (54%)	25 (46%)	
High				24 (45%)	29 (55%)		25 (47%)	28 (53%)	
dCK			0.336						0.442
Low	30 (56%)	24 (44%)					25 (46%)	29 (54%)	
High	24 (45%)	29 (55%)					29 (55%)	24 (45%)	
HuR C			0.564			0.442			
Low	29 (54%)	25 (46%)		25 (46%)	29 (54%)				
High	25 (47%)	28 (53%)		29 (55%)	24 (45%)				
Year of surgery, M (IQR)	2009 (2005–2010)	2009 (2007–2011)	0.634	2009 (2005–2010)	2009 (2006–2011)	0.783	2008 (2004–2010)	2009 (2007–2010)	0.054
Age, M (IQR)	66 (61–71)	67 (62–73)	0.805	67 (61–73)	66 (62–72)	0.246	67 (61–73)	67 (62–72)	0.177
Sex			0.563			0.123			**0.004**
Women	27 (54%)	23 (46%)		21 (42%)	29 (58%)		33 (66%)	17 (34%)	
Men	27 (47%)	30 (53%)		33 (58%)	24 (42%)		21 (37%)	36 (63%)	
Tumour origin			0.765			0.351			0.623
Ampulla Vateri	11 (58%)	8 (42%)		11 (58%)	8 (42%)		8 (42%)	11 (58%)	
Distal bile duct	21 (47%)	24 (53%)		19 (42%)	26 (58%)		25 (56%)	20 (44%)	
Pancreas	22 (51%)	21 (49%)		24 (56%)	19 (44%)		21 (49%)	22 (51%)	
Tumour size, mm, M (IQR)	30 (25–40)	30 (21–30)	0.349	30 (23–36)	30 (25–35)	0.224	30 (25–35)	30 (22–40)	0.546
Differentiation grade			**0.001**			0.689			0.550
Well/moderate	11 (28%)	28 (72%)		21 (54%)	18 (46%)		18 (46%)	21 (54%)	
Poor	43 (63%)	25 (37%)		33 (49%)	35 (51%)		36 (53%)	32 (47%)	
T-stage			0.556			1.000			1.000
T1/T2	5 (42%)	7 (58%)		6 (50%)	6 (50%)		6 (50%)	6 (50%)	
T3/T4	49 (52%)	46 (48%)		48 (51%)	47 (49%)		48 (51%)	47 (49%)	
N-stage			0.283			0.830			1.000
N0	18 (60%)	12 (40%)		16 (53%)	14 (47%)		15 (50%)	15 (50%)	
N1	36 (47%)	41 (53%)		38 (49%)	39 (51%)		39 (51%)	38 (49%)	
Perineural growth			0.059			0.347			0.812
No	7 (32%)	15 (68%)		9 (41%)	13 (59%)		12 (55%)	10 (45%)	
Yes	47 (55%)	38 (45%)		45 (53%)	40 (47%)		42 (49%)	43 (51%)	
Growth in lymphatic vessels			0.676			0.094			0.528
No	15 (47%)	17 (53%)		12 (38%)	20 (62%)		18 (56%)	14 (44%)	
Yes	39 (52%)	36 (48%)		42 (56%)	33 (44%)		36 (48%)	39 (52%)	
Growth in blood vessels			0.224			0.685			1.000
No	32 (46%)	38 (54%)		34 (49%)	36 (51%)		35 (50%)	35 (50%)	
Yes	22 (59%)	15 (41%)		20 (54%)	17 (46%)		19 (51%)	18 (49%)	
Growth in peripancreatic fat			0.347			0.639			1.000
No	9 (41%)	13 (59%)		10 (45%)	12 (55%)		11 (50%)	11 (50%)	
Yes	45 (53%)	40 (47%)		44 (52%)	41 (48%)		43 (51%)	42 (49%)	
Margins			0.437			0.437			0.678
R0	2 (33%)	4 (67%)		2 (33%)	4 (67%)		4 (67%)	2 (33%)	
R1/Rx	52 (51%)	49 (49%)		52 (51%)	49 (49%)		50 (50%)	51 (50%)	
Adjuvant treatment			0.246			0.847			0.246
No gemcitabine	33 (56%)	26 (44%)		29 (49%)	30 (51%)		33 (56%)	26 (44%)	
Gemcitabine	21 (44%)	27 (56%)		25 (52%)	23 (48%)		21 (44%)	27 (56%)	
Recurrence			0.354			0.507			0.354
None	9 (47%)	10 (53%)		11 (58%)	8 (42%)		9 (47%)	10 (53%)	
Local	18 (62%)	11 (38%)		12 (41%)	17 (59%)		18 (62%)	11 (38%)	
Distant	27 (46%)	32 (54%)		31 (53%)	28 (47%)		27 (46%)	32 (54%)	

IQR, interquartile range; M, median. Bold text indicates significant values.

Table II. Associations between hENT1, dCK, HuR and clinicopathological parameters in intestinal type periampullary adenocarcinomas.

	hENT1			dCK			HuR		
	Low ($n = 32$)	High ($n = 31$)	*p*-value	Low ($n = 32$)	High ($n = 31$)	*p*-value	Low ($n = 33$)	High ($n = 30$)	*p*-value
Excluded from survival analysis	1	1		2			1	1	
hENT1						0.466			**0.021**
Low				17 (55%)	14 (45%)		21 (68%)	10 (32%)	
High				13 (43%)	17 (57%)		11 (37%)	19 (63%)	
dCK			0.466						0.611
Low	17 (57%)	13 (43%)					17 (57%)	13 (43%)	
High	14 (45%)	17 (55%)					15 (48%)	16 (52%)	
HuR			**0.021**			0.611			
Low	21 (66%)	11 (34%)		17 (53%)	15 (47%)				
High	10 (34%)	19 (66%)		13 (45%)	16 (55%)				
Year of surgery, M (IQR)	2007 (2003–2009)	2007 (2003–2010)	0.969	2006 (2002–2009)	2007 (2005–2010)	0.140	2008 (2005–2010)	2005 (2002–2010)	0.659
Age, M (IQR)	66 (59–69)	67 (60–72)	0.372	67 (62–72)	65 (56–70)	0.137	67 (61–70)	66 (59–72)	0.140
Sex			1.000			0.616			0.317
Women	17 (50%)	17 (50%)		16 (47%)	18 (53%)		20 (59%)	14 (41%)	
Men	15 (52%)	14 (48%)		16 (55%)	13 (45%)		13 (45%)	16 (55%)	
Tumour origin			**0.016**			1.000			0.068
Duodenum	3 (21%)	11 (79%)		7 (50%)	7 (50%)		4 (29%)	10 (71%)	
Ampulla Vateri	29 (59%)	20 (41%)		25 (51%)	24 (49%)		29 (59%)	20 (41%)	
Tumour size, mm, M (IQR)	28 (14–35)	27 (15–45)	**0.019**	30 (16–40)	25 (15–40)	0.683	25 (15–40)	30 (15–45)	0.331
Differentiation grade			0.454			0.617			0.133
Well/moderate	14 (45%)	17 (55%)		17 (55%)	14 (45%)		13 (42%)	18 (58%)	
Poor	18 (56%)	14 (44%)		15 (47%)	17 (53%)		20 (63%)	12 (37%)	
T-stage			0.774			1.000			0.769
T1/T2	7 (47%)	8 (53%)		8 (53%)	7 (47%)		7 (47%)	8 (53%)	
T3/T4	25 (52%)	23 (48%)		24 (50%)	24 (50%)		26 (54%)	22 (46%)	
N-stage			0.629			0.665			1.000
N0	15 (45%)	18 (55%)		16 (48%)	17 (52%)		17 (52%)	16 (48%)	
N1	10 (53%)	9 (47%)		9 (47%)	10 (53%)		10 (53%)	9 (47%)	
N2	7 (64%)	4 (36%)		7 (64%)	4 (36%)		6 (55%)	5 (45%)	
Perineural growth			0.585			1.000			**0.031**
No	21 (48%)	23 (52%)		22 (50%)	22 (50%)		19 (43%)	25 (57%)	
Yes	11 (58%)	8 (42%)		10 (53%)	9 (47%)		14 (74%)	5 (26%)	
Growth in lymphatic vessels			**0.023**			0.210			0.133
No	10 (34%)	19 (66%)		12 (41%)	17 (59%)		12 (41%)	17 (59%)	
Yes	22 (65%)	12 (35%)		20 (59%)	14 (41%)		21 (62%)	13 (38%)	
Growth in blood vessels			1.000			0.355			0.054
No	29 (50%)	29 (50%)		28 (48%)	30 (52%)		28 (48%)	30 (52%)	
Yes	3 (60%)	2 (40%)		4 (80%)	1 (20%)		5 (100%)	0 (0%)	
Growth in peripancreatic fat			0.430			0.188			0.111
No	19 (46%)	22 (54%)		18 (44%)	23 (56%)		18 (44%)	23 (56%)	
Yes	13 (59%)	9 (41%)		14 (64%)	8 (36%)		15 (68%)	7 (32%)	
Margins			0.164			**0.050**			0.395
R0	6 (35%)	11 (65%)		5 (29%)	12 (71%)		7 (41%)	10 (59%)	
R1/Rx	26 (57%)	20 (43%)		27 (59%)	19 (41%)		26 (57%)	20 (43%)	
Adjuvant treatment			0.585			1.000			1.000
None	24 (53%)	21 (47%)		23 (51%)	22 (49%)		24 (53%)	21 (47%)	
Any	8 (44%)	10 (56%)		9 (50%)	9 (50%)		9 (50%)	9 (50%)	
Recurrence			0.022			0.125			0.145
None	12 (35%)	22 (65%)		15 (44%)	19 (56%)		14 (41%)	20 (59%)	
Local	3 (75%)	1 (25%)		4 (100%)	0 (0%)		2 (50%)	2 (50%)	
Distant	17 (68%)	8 (32%)		13 (52%)	12 (48%)		17 (68%)	8 (32%)	

IQR, interquartile range; M, median. Bold text indicates significant values.

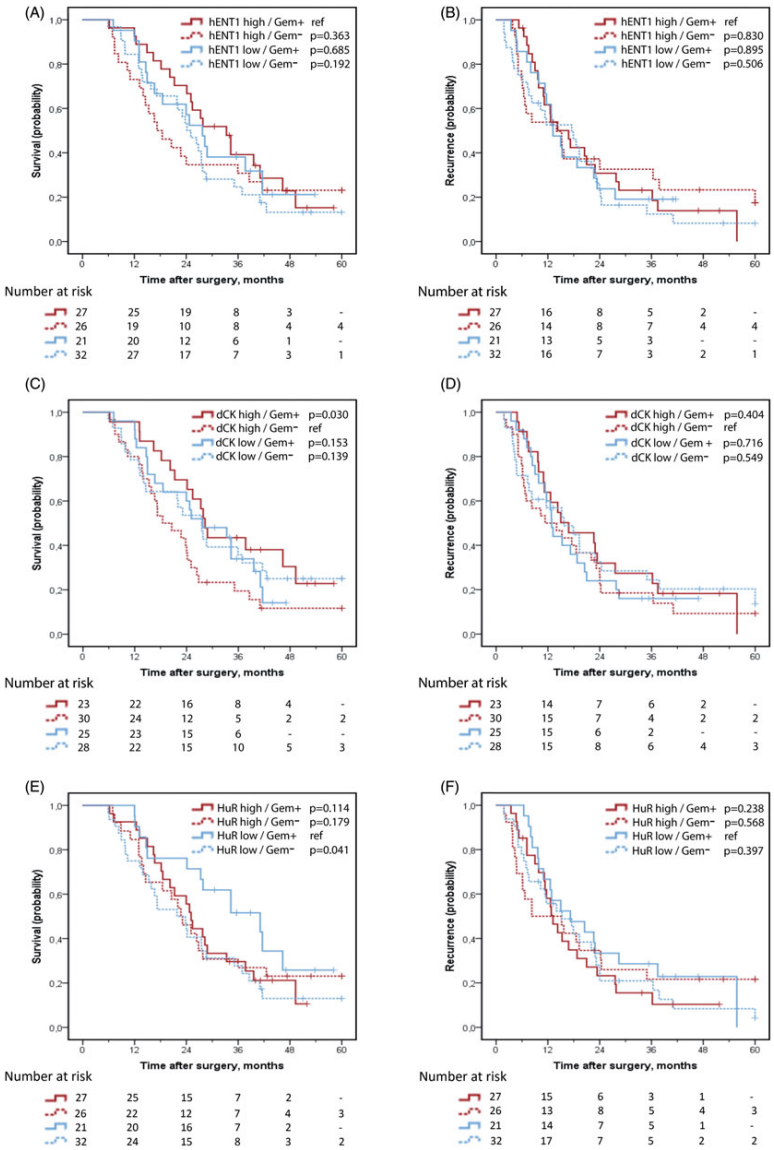


Figure 2. Kaplan–Meier curves of overall survival and recurrence-free survival in pancreatobiliary type tumours stratified by hENT1 (A,B), dCK (C,D) and HuR (E,F) expression and adjuvant gemcitabine.

(Table IV) and OS (Supplementary Table III, available online at http://www.informahealthcare.com).

High HuR expression was associated with a significantly longer OS in the entire group of patients with I-type tumours, and also in patients not receiving adjuvant chemotherapy (Figure 3E and F). These findings were confirmed in univariable analysis for RFS in the entire group (HR 0.41, 95% CI 0.19–0.88) and in patients not receiving adjuvant chemotherapy (HR 0.37, 95% CI 0.15–0.92) (Table IV). Similar results were seen for OS (Supplementary Table III). Significance was however not

Table III. Cox proportional hazards analysis of the impact of expression of hENT1, dCK, HuR and HuR cytoplasmic/nuclear ratio on recurrence-free survival in patients with pancreatobiliary type tumours.

	Number (events)	RFS HR (95% CI) Unadjusted	Adjusted	*P* for interaction
hENT1				
All				
Low	53 (45)	1.00	1.00	
High	53 (43)	0.86 (0.56–1.31)	1.35 (0.83–2.20)	
No gemcitabine				*NS*
Low	32 (28)	1.00	1.00	
High	26 (20)	0.79 (0.44–1.41)	1.18 (0.62–2.22)	
Gemcitabine				
Low	21 (17)	1.00	1.00	
High	27 (23)	0.96 (0.51–1.81)	1.87 (0.92–3.83)	
dCK				
All				
Low	53 (43)	1.00	1.00	
High	53 (45)	1.03 (0.68–1.57)	1.02 (0.65–1.59)	
No gemcitabine				*NS*
Low	28 (22)	1.00	1.00	
High	30 (26)	1.19 (0.67–2.11)	0.85 (0.43–1.69)	
Gemcitabine				
Low	25 (21)	1.00	1.00	
High	23 (19)	0.80 (0.42–1.50)	0.97 (0.49–1.94)	
HuR				
All				
Low	53 (45)	1.00	1.00	
High	53 (43)	1.07 (0.70–1.63)	1.30 (0.84–2.00)	
No gemcitabine				*NS*
Low	32 (28)	1.00	1.00	
High	26 (20)	0.87 (0.49–1.56)	1.00 (0.53–1.87)	
No Gemcitabine				
Low	21 (17)	1.00	1.00	
High	27 (23)	1.47 (0.77–2.79)	1.51 (0.79–2.90)	
HuR C/N ratio				
All				
Low	53 (44)	1.00	1.00	
High	53 (44)	1.04 (0.68–1.58)	1.31 (0.84–2.04)	
No gemcitabine				*0.053*
Low	30 (27)	1.00	1.00	
High	28 (21)	0.72 (0.40–1.27)	0.78 (0.42–1.46)	
Gemcitabine				
Low	23 (17)	1.00	1.00	
High	25 (23)	1.59 (0.84–3.01)	**2.59 (1.29–5.20)**	

The multivariable model included age (continuous), T-stage (1–2 vs. 3–4), N-Stage, differentiation grade (well-moderate vs. poor), lymphatic invasion, vascular invasion, perineural growth, and in the analysis including all cases also gemcitabine treatment (yes/no). C/N ratio, cytoplasmic/nuclear ratio; NS, non-significant. Bold text indicates significant values.

retained in multivariable analysis neither for RFS (Table IV) nor OS (Supplementary Table III). A high HuR C/N ratio was also significantly associated with a longer OS and borderline significantly associated with a prolonged RFS, which was confirmed in univariable analysis for RFS (HR 0.47, 95% CI 0.22–1.02) (Table IV) and OS (Supplementary Table III). The associations were significant in multivariable analysis for OS (Supplementary Table III), but not for RFS (Table IV).

Potential predictive value of hENT1, dCK and HuR expression

In patients with PB-type tumours receiving adjuvant gemcitabine, a high HuR C/N ratio was significantly associated with a reduced OS in univariable analysis (HR 2.07, 95% CI 1.03–4.17), with a significant interaction ($p_{interaction}$=0.028) (Supplementary Table II). For RFS, there was a borderline significant treatment interaction ($p_{interaction}$=0.053) (Table III).

There was no significant treatment interaction for hENT1, dCK or cytoplasmic HuR expression with regard to RFS or OS (Table III and Supplementary Table II).

In I-type tumours, high dCK expression was significantly associated with a prolonged RFS in patients receiving adjuvant chemotherapy (univariable HR 0.09, 95% CI 0.01–0.73), with a significant treatment interaction ($p_{interaction}$=0.023) (Table IV). Cox regression and interaction analysis could not be performed for dCK with regard to OS, as there were no fatalities among the nine patients with high dCK expression having received adjuvant chemotherapy.

The prognostic value of hENT1, HuR, or HuR C/N in I-type tumours did not differ by adjuvant treatment, neither for RFS (Table IV) nor OS (Supplementary Table III).

Discussion

In the group of PB-type tumours, including pancreatic cancer, our results do not support previous results in large cohorts on the predictive value of high hENT1 expression [2,4]. Our results on high dCK expression in relation to gemcitabine response are however in line with previous findings [2]. Our results do not confirm the previously described association between HuR and dCK expression described in 116 cases of pancreatic cancer [9] and in cell lines [13]. Moreover, our results regarding the predictive value of HuR, with a better survival in patients having received gemcitabine with tumours displaying low expression of HuR or a low C/N ratio, differ from previous reports on two smaller series of gemcitabine-treated patients with pancreatic cancer (n=32 and n=24, respectively), where high HuR expression was found to be associated with a prolonged survival [13,14]. Our results are however plausible, as HuR increases proteins related to proliferation, angiogenesis and evasion of apoptosis, thus promoting a more malignant phenotype [10,11]. The findings of an association between high HuR or a high HuR C/N ratio and a poorer prognosis also harmonise with a majority of reports on HuR in different tumour types, where a high cytoplasmic expression of HuR or a high C/N ratio were found to confer a worse prognosis [12].

In the group of I-type periampullary adenocarcinomas, expression of dCK was found to be potentially predictive of response to adjuvant chemotherapy, which has, to the best of our knowledge, not been described before. Although several of these patients had received adjuvant gemcitabine, there are indications that dCK also increases sensitivity to 5-FU [9].

Our findings on HuR in I-type tumours are more surprising, with high expression being significantly associated with a better prognosis, regardless of treatment. I-type periampullary tumours are often assumed to behave similarly to colorectal or gastric cancer, but our results on HuR differ from previous reports on these tumour types [15,21], and also deviate from the concept of HuR being a positive regulator of malignant behaviour in other tumour types [12]. In the herein investigated tumours, perineural growth was less common in I-type tumours with high HuR expression, which is in line with its beneficial impact on survival. Whether the distribution of perineural growth in the groups of high or low HuR is coincidental or biologically related to levels of HuR cannot be

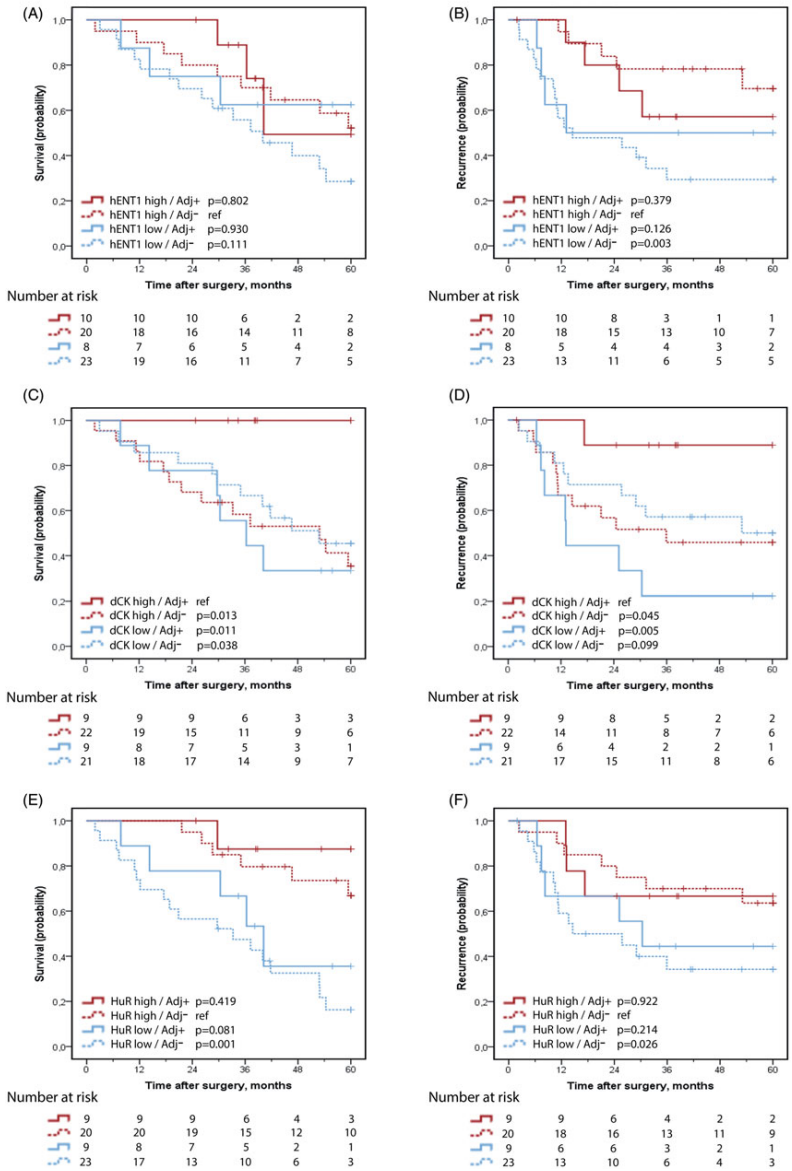


Figure 3. Kaplan–Meier curves of overall survival and recurrence-free survival in intestinal type tumours, stratified by hENT1 (A,B), dCK (C,D) and HuR (E,F) expression and adjuvant chemotherapy.

determined based on the results from this study, but the non-significant hazard ratio for HuR in multivariable analysis indicates that its associations with other parameters may explain its prognostic effect in I-type tumours.

High hENT1 expression was more common in I-type tumours of duodenal origin than of ampullary origin, which could explain its association with a more favourable prognosis. A borderline significant association between a longer RFS

Table IV. Cox proportional hazards analysis of the impact of expression of hENT1, dCK, HuR and HuR cytoplasmic/nuclear ratio on recurrence-free survival in patients with intestinal type tumours.

		RFS HR (95% CI)		
	Number (events)	Unadjusted	Adjusted	*p* for interaction
hENT1				
All				
Low	31 (20)	1.00	1.00	
High	30 (9)	**0.33 (0.15–0.72)**	**0.24 (0.10–0.59)**	
No adjuvant				*NS*
Low	23 (16)	1.00	1.00	
High	20 (5)	**0.24 (0.09–0.67)**	**0.07 (0.02–0.28)**	
Adjuvant				
Low	8 (4)	1.00		
High	10 (4)	0.59 (0.15–2.39)	†	
dCK				
All				
Low	30 (17)	1.00	1.00	
High	31 (12)	0.68 (0.33–1.43)	0.82 (0.38–1.76)	
No adjuvant				*0.023*
Low	21 (10)	1.00	1.00	
High	22 (11)	1.26 (0.53–2.97)	1.56 (0.61–4.02)	
Adjuvant				
Low	9 (7)	1.00		
High	9 (1)	**0.09 (0.01–0.73)**	†	
HuR				
All				
Low	32 (19)	1.00	1.00	
High	29 (10)	**0.41 (0.19–0.88)**	0.47 (0.21–1.04)	
No adjuvant				*NS*
Low	23 (14)	1.00	1.00	
High	20 (7)	**0.37 (0.15–0.92)**	0.46 (0.16–1.32)	
Adjuvant				
Low	9 (5)	1.00		
High	9 (3)	0.52 (0.12–2.21)	†	
HuR C/N ratio				
All				
Low	32 (19)	1.00	1.00	
High	29 (10)	0.47 (0.22–1.02)	0.44 (0.19–1.02)	
No adjuvant				*NS*
Low	24 (15)	1.00	1.00	
High	19 (6)	**0.39 (0.15–1.00)**	**0.14 (0.03–0.62)**	
Adjuvant				
Low	8 (4)	1.00		
High	10 (4)	0.80 (0.20–3.23)	†	

The multivariable model included age (continuous), T-stage (1–2 vs 3–4), N-Stage, differentiation grade (well-moderate vs. poor), lymphatic invasion, vascular invasion, perineural growth, and in the analysis including all cases also adjuvant treatment (yes/no). Dagger (†) indicates that multivariable analysis was not performed due to few cases and events. C/N ratio, cytoplasmic/nuclear ratio; NS, non-significant. Bold text indicates significant values.

and high hENT1 was however retained in subgroup analysis of I-type cases of ampullary origin. Invasion of lymphatic vessels was less common in cases displaying high hENT1 expression, but the significant association between high hENT1 and RFS was retained in multivariable analysis, adjusting for growth in lymphatic vessels. These findings are in contrast with previous reports on ampullary and gastric cancer, where hENT1 expression was demonstrated to be associated with a shorter survival [6,22].

The associations between HuR and hENT1 in I-type tumours and clinicopathological parameters also illustrate the risk for type I errors when the number of correlation tests are many, and should thus be evaluated with caution.

The hENT1 antibody used in the present study has been validated in a study by Poplin et al. [23] against a different, not commercially available, antibody (10D7G2) used, e.g. in the studies by Farrell et al. and Maréchal et al. [2,3]. To this end, tumour samples from the RTOG [3] study were independently stained and analysed with the SP120 antibody on newly constructed TMAs, with concordant results [23]. Of note, the aim of the study by Poplin et al. was to evaluate hENT1 expression prospectively in order to compare the efficacy of gemcitabine with CO-101, a lipid-drug conjugate of gemcitabine. According to the results, based on analyses of metastatic lesions, CO-101 was not demonstrated to be superior to gemcitabine in patients with low tumour-specific hENT1 expression and hENT1 expression did not predict survival within the gemcitabine arm [23].

We are not aware of any previous studies comparing the expression of the herein investigated biomarkers in primary tumours and paired lymph node metastases. Our results demonstrate a significantly increased expression from primary tumour to metastasis of both dCK and hENT1 in PB-type tumours. The potential mechanistic basis for this observation remains unclear, but may however have implications in the clinical setting, i.e. that biomarker assessment in metastatic components may be sufficient when the primary tumour is not available for analysis, i.e. in the palliative setting.

The cohort used in this study is well characterised regarding clinicopathological parameters, and follow-up, and adjuvant chemotherapy has only been given to approximately half of the patients, which enables a fairly good assessment of both prognostic and potentially predictive biomarkers even in the retrospective setting. Limitations due to the size of the cohort are mostly seen in I-type tumours, in particular when stratifying both for biomarker expression and adjuvant treatment. Still, similar results regarding the predictive effect of dCK as described by others in pancreatic cancer was seen in both PB- and I-type tumours.

In conclusion, the results from the present study demonstrate that hENT1 expression is a favourable prognostic factor in patients with I-type, but not in PB-type tumours, and not potentially response predictive in neither morphological subtype. Moreover, a high cytoplasmic/nuclear HuR ratio was found to be a negative prognostic factor in patients with PB-type tumours receiving adjuvant gemcitabine, and high dCK expression was found to be a positive prognostic factor in patients with I-type tumours receiving any adjuvant treatment. The finding regarding dCK expression in I-type tumours is novel and of potential clinical relevance, and therefore merits further study, preferably in tumours from randomised, prospective trials. These findings also highlight the importance of taking morphological subtype into consideration in biomarker studies related to periampullary cancer.

Acknowledgements

This study was supported by grants from the Knut and Alice Wallenberg Foundation, the Swedish Cancer Society, the Mrs Berta Kamprad Foundation, the Gunnar Nilsson Cancer Foundation, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and University Hospital Research Grants.

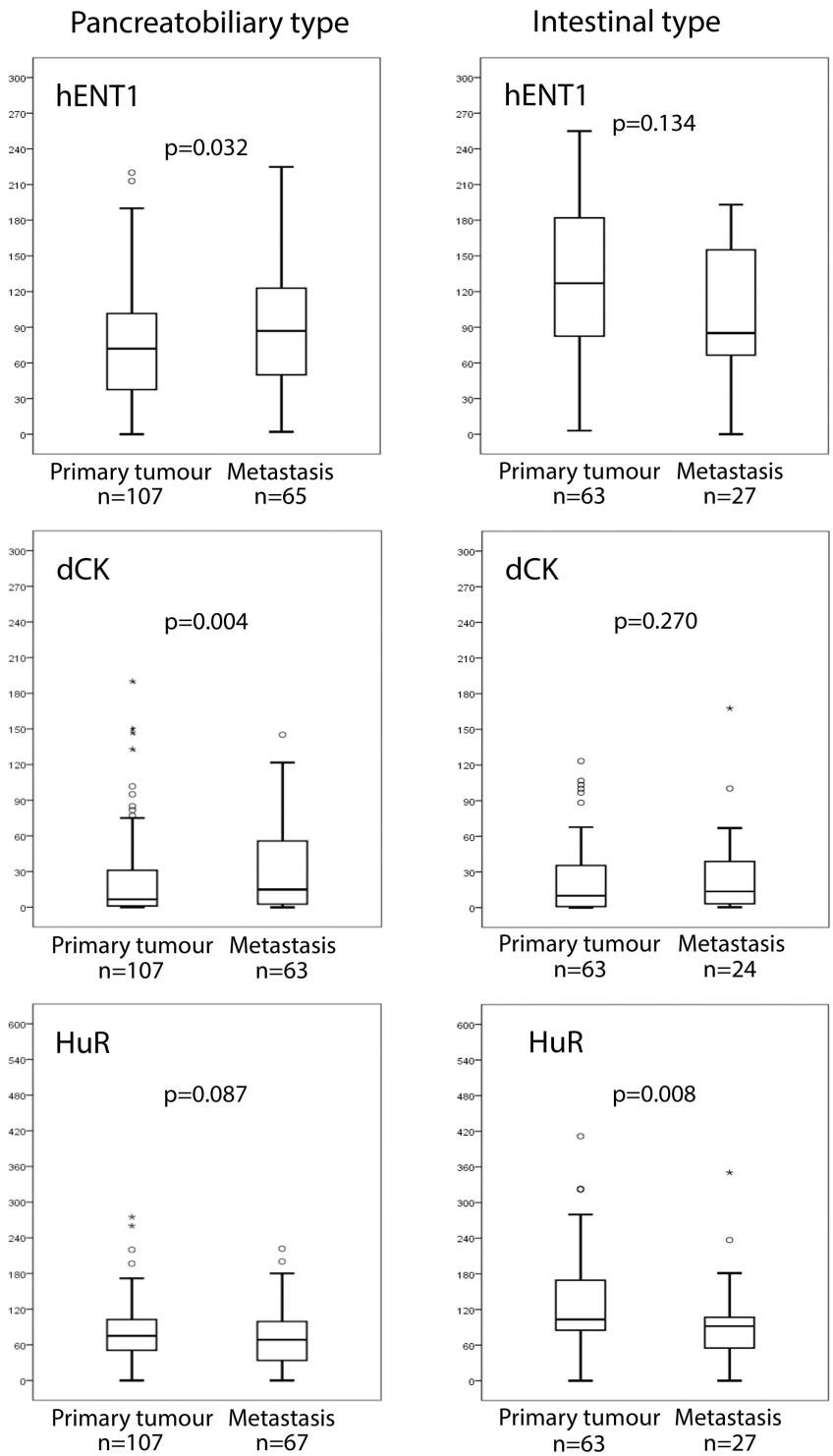
Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

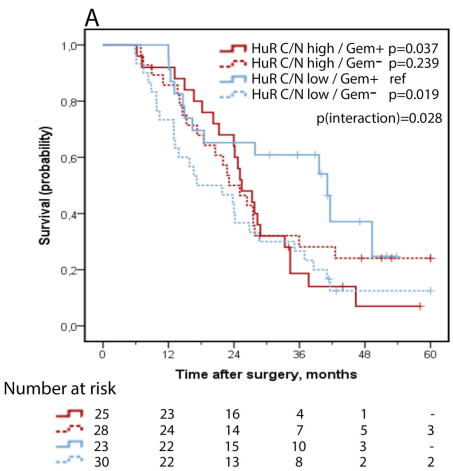
1. Wei CH, Gorgan TR, Elashoff DA, Hines OJ, Farrell JJ, Donahue TR. A meta-analysis of gemcitabine biomarkers in patients with pancreaticobiliary cancers. Pancreas 2013;42:1303–10.
2. Marechal R, Bachet JB, Mackey JR, Dalban C, Demetter P, Graham K, et al. Levels of gemcitabine transport and metabolism proteins predict survival times of patients treated with gemcitabine for pancreatic adenocarcinoma. Gastroenterology 2012;143:664–74e1–6.
3. Farrell JJ, Elsaleh H, Garcia M, Lai R, Ammar A, Regine WF, et al. Human equilibrative nucleoside transporter 1 levels predict response to gemcitabine in patients with pancreatic cancer. Gastroenterology 2009;136:187–95.
4. Greenhalf W, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, Lamb RF, et al. Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. J Natl Cancer Inst 2014;106:djt347.
5. Perrone G, Morini S, Santini D, Rabitti C, Vincenzi B, Alloni R, et al. Human equilibrative nucleoside transporter 1 and carcinoma of the ampulla of Vater: Expression differences in tumour histotypes. Eur J Histochem 2010;54:e38.
6. Santini D, Perrone G, Vincenzi B, Lai R, Cass C, Alloni R, et al. Human equilibrative nucleoside transporter 1 (hENT1) protein is associated with short survival in resected ampullary cancer. Ann Oncol 2008;19:724–8.
7. Bouffard DY, Laliberte J, Momparler RL. Kinetic studies on 2′,2′-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem Pharmacol 1993;45:1857–61.
8. Saiki Y, Yoshino Y, Fujimura H, Manabe T, Kudo Y, Shimada M, et al. DCK is frequently inactivated in acquired gemcitabine-resistant human cancer cells. Biochem Biophys Res Commun 2012;421:98–104.
9. McAllister F, Pineda DM, Jimbo M, Lal S, Burkhart RA, Moughan J, et al. dCK expression correlates with 5-fluorouracil efficacy and HuR cytoplasmic expression in pancreatic cancer: A dual-institutional follow-up with the RTOG 9704 trial. Cancer Biol Ther 2014;15:688–98.
10. Hinman MN, Lou H. Diverse molecular functions of Hu proteins. Cell Mol Life Sci 2008;65:3168–81.
11. Lopez de Silanes I, Lal A, Gorospe M. HuR: Post-transcriptional paths to malignancy. RNA Biol 2005;2:11–13.
12. Kotta-Loizou I, Giaginis C, Theocharis S. Clinical significance of HuR expression in human malignancy. Med Oncol 2014;31:161:1–19
13. Costantino CL, Witkiewicz AK, Kuwano Y, Cozzitorto JA, Kennedy EP, Dasgupta A, et al. The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR Up-regulates the expression of the gemcitabine metabolizing enzyme deoxycytidine kinase. Cancer Res 2009;69:4567–72.
14. Richards NG, Rittenhouse DW, Freydin B, Cozzitorto JA, Grenda D, Rui H, et al. HuR status is a powerful marker for prognosis and response to gemcitabine-based chemotherapy for resected pancreatic ductal adenocarcinoma patients. Ann Surg 2010;252:499–505; discussion –6.
15. Yoo PS, Sullivan CA, Kiang S, Gao W, Uchio EM, Chung GG, et al. Tissue microarray analysis of 560 patients with colorectal adenocarcinoma: High expression of HuR predicts poor survival. Ann Surg Oncol 2009;16:200–7.
16. Elebro J, Jirstrom K. Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma. Diagn Pathol 2014;9:80:1–10
17. Elebro J, Heby M, Gaber A, Nodin B, Jonsson L, Fristedt R, et al. Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. J Transl Med 2014; 12:289:1–115.
18. Fristedt R, Elebro J, Gaber A, Jonsson L, Heby M, Yudina Y, et al. Reduced expression of the polymeric immunoglobulin receptor in pancreatic and periampullary adenocarcinoma signifies tumour progression and poor prognosis. PLoS One 2014;9:e112728.
19. Heby M, Elebro J, Nodin B, Jirstrom K, Eberhard J. Prognostic and predictive significance of podocalyxin-like protein expression in pancreatic and periampullary adenocarcinoma. BMC Clin Pathol 2015;15:10.
20. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
21. Mrena J, Wiksten JP, Thiel A, Kokkola A, Pohjola L, Lundin J, et al. Cyclooxygenase-2 is an independent prognostic factor in gastric cancer and its expression is regulated by the messenger RNA stability factor HuR. Clin Cancer Res 2005;11:7362–8.
22. Santini D, Vincenzi B, Fratto ME, Perrone G, Lai R, Catalano V, et al. Prognostic role of human equilibrative transporter 1 (hENT1) in patients with resected gastric cancer. J Cell Physiol 2010;223:384–8.
23. Poplin E, Wasan H, Rolfe L, Raponi M, Ikdahl T, Bondarenko I, et al. Randomized, multicenter, phase II study of CO-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: Including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. J Clin Oncol 2013;31:4453–61.

Supplementary material available online
Supplementary Figures 1 and 2, Tables 1–3

Supplementary Figure 1

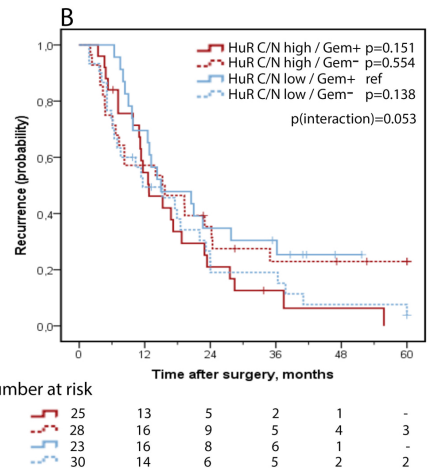


Supplementary Figure 2



Number at risk

HuR C/N high / Gem+	25	23	16	4	1	-
HuR C/N high / Gem-	28	24	14	7	5	3
HuR C/N low / Gem+	23	22	15	10	3	-
HuR C/N low / Gem-	30	22	13	8	2	2



Number at risk

HuR C/N high / Gem+	25	13	5	2	1	-
HuR C/N high / Gem-	28	16	9	5	4	3
HuR C/N low / Gem+	23	16	8	6	1	-
HuR C/N low / Gem-	30	14	6	5	2	2

Supplementary table 1

Supplementary table 1. Associations between HuR C/N ratio and clinicopathological parameters in pancreatobiliary adenocarcinomas

	HuR C/N ratio		
	low (n=54)	high (n=55)	p-value
Excluded, neoadjuvant treatment		2	
Lost to follow up	1		
hENT1			0.564
low	29 (54%)	25 (46%)	
high	25 (47%)	28 (53%)	
dCK			0.442
low	25 (46%)	29 (54%)	
high	29 (55%)	24 (45%)	
HuR C			**<0.001**
low	40 (74%)	14 (26%)	
high	14 (26%)	39 (74%)	
Year of surgery, M (IQR)	2010 (2005-2011)	2008 (2006-2010)	0.456
Age, M (IQR)	66 (61-73)	67 (62-72)	0.469
Sex			**0.001**
Women	34 (68%)	16 (32%)	
Men	20 (35%)	37 (65%)	
Tumour origin			0.051
Ampulla Vateri	5 (26%)	14 (74%)	
Distal bile duct	27 (60%)	18 (40%)	
Pancreas	22 (51%)	21 (49%)	
Tumour size, mm, M (IQR)	30 (24-35)	30 (23-38)	0.985
Differentiation grade			0.072
Well / moderate	15 (38%)	24 (62%)	
Poor	39 (57%)	29 (43%)	
T-stage			1.000
T1 / T2	6 (50%)	6 (50%)	
T3 / T4	48 (51%)	47 (49%)	
N-stage			1.000
N0	15 (50%)	15 (50%)	
N1	39 (51%)	38 (49%)	
Perineural growth			0.347
No	9 (41%)	13 (59%)	
Yes	45 (53%)	40 (47%)	
Growth in lymphatic vessels			0.140
No	20 (63%)	12 (37%)	
Yes	34 (45%)	41 (55%)	
Growth in blood vessels			0.546
No	37 (53%)	33 (47%)	
Yes	17 (46%)	20 (54%)	
Growth in peripancreatic fat			0.474
No	13 (59%)	9 (41%)	
Yes	41 (48%)	44 (52%)	
Margins			**0.027**
R0	6 (100%)	0 (0%)	
R1/Rx	48 (48%)	53 (52%)	
Adjuvant treatment			0.699
No gemcitabine	31 (52%)	28 (47%)	
Gemcitabine	23 (48%)	25 (52%)	
Recurrence			0.294
None	10 (53%)	9 (47%)	
Local	18 (62%)	11 (38%)	
Distant	26 (44%)	33 (56%)	

M, median. IQR, interquartile range. Bold text indicates significant values.

Supplementary table 2

Supplementary table 2. Cox proportional hazards analysis of the impact of expression of hENT1, dCK, HuR and HuR cytoplasmic/nuclear ratio on overall survival in patients with pancreatobiliary type tumours

	Number (events)	**OS HR (95% CI)**		***P for interaction***
		unadjusted	adjusted	
hENT1				
All				
Low	53 (42)	1.00	1.00	
High	53 (40)	0.89 (0.57-1.37)	1.59 (0.97-2.61)	
No gemcitabine				
Low	32 (27)	1.00	1.00	
High	26 (20)	0.98 (0.55-1.76)	**2.20 (1.12-4.30)**	*NS*
Gemcitabine				
Low	21 (15)	1.00	1.00	
High	27 (20)	0.87 (0.44-1.71)	1.46 (0.69-3.08)	
dCK				
All				
Low	53 (40)	1.00	1.00	
High	53 (42)	1.15 (0.75-1.78)	1.20 (0.77-1.87)	
No gemcitabine				
Low	28 (21)	1.00	1.00	
High	30 (26)	1.55 (0.86-2.79)	**1.89 (1.03-3.46)**	*NS*
Gemcitabine				
Low	25 (19)	1.00	1.00	
High	23 (16)	0.71 (0.36-1.42)	0.69 (0.35-1.37)	
HuR				
All				
Low	53 (40)	1.00	1.00	
High	53 (42)	1.09 (0.70-1.68)	1.16 (0.75-1.80)	
No Gemcitabine				
Low	32 (27)	1.00	1.00	
High	26 (20)	0.83 (0.46-1.48)	0.81 (0.44-1.50)	*NS*
Gemcitabine				
Low	21 (13)	1.00	1.00	
High	27 (22)	1.74 (0.87-3.47)	1.62 (0.81-3.26)	
HuR C/N ratio				
All				
Low	53 (39)	1.00	1.00	
High	53 (43)	1.09 (0.71-1.69)	1.07 (0.68-1.67)	
No Gemcitabine				
Low	30 (26)	1.00	1.00	
High	28 (21)	0.72 (0.40-1.28)	0.56 (0.31-1.01)	***0.028***
Gemcitabine				
Low	23 (13)	1.00	1.00	
High	25 (22)	**2.07 (1.03-4.17)**	**2.19 (1.08-4.45)**	

The multivariable model included age (continuous), T-stage (1-2 vs 3-4), N-Stage, differentiation grade (well-moderate vs poor), lymphatic invasion, vascular invasion, perineural growth, and in the analysis including all cases also gemcitabine treatment (yes/no). C/N ratio= cytoplasmic/nuclear ratio. Bold text indicates significant values. NS= non-significant

Supplementary table 3

Supplementary table 3. Cox proportional hazards analysis of the impact of expression of hENT1, dCK, HuR and HuR cytoplasmic/nuclear ratio on overall survival in patients with intestinal type tumours

	Number (events)	OS HR (95% CI)		*P for interaction*
		unadjusted	adjusted	
hENT1				
All				
Low	31 (18)	1.00	1.00	
High	30 (12)	0.57 (0.28-1.19)	0.49 (0.22-1.11)	
No adjuvant				
Low	23 (15)	1.00	1.00	
High	20 (9)	0.51 (0.22-1.18)	**0.33 (0.12-0.85)**	
Adjuvant				*NS*
Low	8 (3)	1.00		
High	10 (3)	0.82 (0.16-4.11)	†	
dCK				
All				
Low	30 (17)	1.00	1.00	
High	31 (13)	0.78 (0.38-1.61)	1.09 (0.50-2.33)	
No adjuvant				
Low	21 (11)	1.00	1.00	
High	22 (13)	1.28 (0.57-2.86)	1.54 (0.65-3.64)	*
Adjuvant				
Low	9 (6)			
High	9 (0)	*	*	
HuR				
All				
Low	32 (23)	1.00	1.00	
High	29 (7)	**0.21 (0.09-0.49)**	**0.26 (0.11-0.64)**	
No adjuvant				
Low	23 (18)	1.00	1.00	
High	20 (6)	**0.22 (0.09-0.56)**	**0.23 (0.09-0.60)**	
Adjuvant				*NS*
Low	9 (5)	1.00		
High	9 (1)	0.18 (0.02-1.51)	†	
HuR C/N ratio				
All				
Low	32 (20)	1.00	1.00	
High	29 (10)	**0.42 (0.20-0.91)**	**0.42 (0.19-0.93)**	
No adjuvant				
Low	24 (16)	1.00	1.00	
High	19 (8)	0.50 (0.21-1.18)	0.40 (0.16-1.01)	
Adjuvant				*NS*
Low	8 (4)	1.00		
High	10 (2)	0.31 (0.05-1.83)	†	

The multivariable model included age (continuous), T-stage (1-2 vs 3-4), N-Stage, differentiation grade (well-moderate vs poor), lymphatic invasion, vascular invasion, perineural growth, and in the analysis including all cases also adjuvant treatment (yes/no). C/N ratio= cytoplasmic/nuclear ratio. Bold text indicates significant values. NS= non-significant. Asterisk (*) indicates non-computable HR and interaction, due to no events in one stratum. Dagger (†) indicates that multivariable analysis was not performed due to few cases and events.

Paper IV

PLOS | ONE

RESEARCH ARTICLE

Expression and Prognostic Significance of Human Epidermal Growth Factor Receptors 1, 2 and 3 in Periampullary Adenocarcinoma

Jacob Elebro*, Margareta Heby, Carl Fredrik Warfvinge, Björn Nodin, Jakob Eberhard, Karin Jirström

Department of Clinical Sciences, Division of Oncology and Pathology, Lund University, Skåne University Hospital, 221 85, Lund, Sweden

* jacob.elebro@med.lu.se

Citation: Elebro J, Heby M, Warfvinge CF, Nodin B, Eberhard J, Jirström K (2016) Expression and Prognostic Significance of Human Epidermal Growth Factor Receptors 1, 2 and 3 in Periampullary Adenocarcinoma. PLoS ONE 11(4): e0153533. doi:10.1371/journal.pone.0153533

Editor: Yves St-Pierre, INRS, CANADA

Received: February 6, 2016

Accepted: March 30, 2016

Published: April 12, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by grants from the Swedish Research Council, the Swedish Cancer Society, the Gunnar Nilsson Cancer Foundation, the Mrs Berta Kamprad Foundation, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and University Hospital Research Grants. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Periampullary adenocarcinoma, including pancreatic cancer, is a heterogeneous group of tumours with dismal prognosis, for which there is an urgent need to identify novel treatment strategies. The human epithelial growth factor receptors EGFR, HER2 and HER3 have been studied in several tumour types, and HER-targeting drugs have a beneficial effect on survival in selected types of cancer. However, these effects have not been evident in pancreatic cancer, and remain unexplored in other types of periampullary cancer. The prognostic impact of HER-expression in these cancers also remains unclear. The aim of this study was therefore to examine the expression and prognostic value of EGFR, HER2 and HER3 in periampullary cancer, with particular reference to histological subtype. To this end, protein expression of EGFR, HER2 and HER3, and *HER2* gene amplification was assessed by immunohistochemistry and silver *in situ* hybridization, respectively, on tissue microarrays with tumours from 175 periampullary adenocarcinomas, with follow-up data on recurrence-free survival (RFS) and overall survival (OS) for up to 5 years. EGFR expression was similar in pancreatobiliary (PB) and intestinal (I) type tumours, but high HER2 and HER3 expression was significantly more common in I-type tumours. In PB-type cases receiving adjuvant gemcitabine, but not in untreated cases, high EGFR expression was significantly associated with a shorter OS and RFS, with a significant treatment interaction in relation to OS ($p_{interaction} = 0.042$). In I-type cases, high EGFR expression was associated with a shorter OS and RFS in univariable, but not in multivariable, analysis. High HER3 expression was associated with a prolonged RFS in univariable, but not in multivariable, analysis. Neither HER2 protein expression nor gene amplification was prognostic. The finding of a potential interaction between the expression of EGFR and response to adjuvant chemotherapy in PB-type tumours needs validation, and merits further study.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Adenocarcinomas originating in the head of the pancreas, the distal bile duct, the ampulla of Vater and the duodenum are often grouped together as periampullary tumours, since they can be difficult to distinguish from each other clinically. Pancreatic cancer is the most common type of periampullary adenocarcinoma, accounting for 3% of all cancer in the USA [1] and the Nordic countries [2] and, due to very high lethality [3], 7% of all cancer-related deaths, making it the fourth most common cause of cancer-related death in the western world [1, 2]. After surgery, periampullary adenocarcinomas have traditionally been primarily categorized according to their anatomical origin, but recent research has shown that morphological subtype is a more rational basis for classification [4]. Pancreatobiliary (PB) type morphology dominates in periampullary tumours of pancreatic and distal bile duct origin, but is also seen in the ampulla of Vater [4, 5]. They have a significantly worse prognosis than tumours with an intestinal (I) type morphology, which are mainly found in the ampulla of Vater and in the duodenum [4–6].

Members of the HER (*human epithelial growth factor receptor)* family of tyrosine kinase receptors are essential for human development and growth, and they are overexpressed in several human cancers. They consist of four closely related transmembranous molecules, EGFR (HER1, ErbB-1), HER2 (Neu, ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). Ligand-binding causes hetero- or homodimerization of receptors and intracellular transphosphorylation, which activates several intracellular signalling-cascades important for cell survival, proliferation and growth. Combinations of HERs give dimers that vary in stability, affinity for their ligands and activation of different signalling-cascades [7].

There are several drugs, targeting either the extracellular domains or the intracellular tyrosine kinase domains of the HERs, that give survival benefits in selected cases of breast, colon, gastric and lung cancer [7], and combinations of HER-active drugs have been shown to further improve survival compared with single HER-therapy [8].

Expression of EGFR is common in pancreatic cancer, and has been associated with metastatic potential [9], but several studies have not found any prognostic effect of EGFR expression on overall survival (OS) [9–11]. A meta-analysis did however find a survival disadvantage in pancreatic cancer expressing EGFR [12]. Addition of the EGFR tyrosine kinase inhibitor erlotinib to gemcitabine lead to an increased OS in patients with advanced pancreatic cancer [13], the improvement was however modest and erlotinib is therefore rarely used for treatment of pancreatic cancer in clinical practice. Other EGFR active drugs have not led to a prolonged OS, when added to standard chemotherapy [7]. EGFR expression has also been shown to be more common in PB-type than in I-type ampullary adenocarcinoma [14] and overexpression has been associated with shorter OS in I-type but not in PB-type tumours [15].

The reported rates of HER2 overexpression in pancreatic cancer, defined as 3+ in immunohistochemical staining or gene amplification by in situ hybridization (ISH), vary from 0%-11% [16–23]. Similarly to other tumour types, high expression of HER2 in pancreatic cancer has been associated with a shorter survival [24], but other studies have found the opposite [25]. Addition of the HER2 antibody trastuzumab to gemcitabine in metastatic pancreatic cancer overexpressing HER2 (2+ or 3+) gave no clear survival benefit compared with the expected survival upon gemcitabine alone [26].

In tumours of the ampulla, distal bile duct and gall bladder, the frequency of HER2 overexpression has been low, and comparable to pancreatic cancer in a few small studies [16, 17, 23], whereas larger studies have found overexpression in 6–13% of ampullary tumours [27, 28], and 23% and 17% of tumours of the bile duct and gall bladder [29].

In ampullary adenocarcinoma, HER2 gene amplification has been equally distributed over morphological types, and amplified cases have been wild-type for KRAS, NRAS and BRAF.

There has also been an equal distribution of 2+ and 3+ immunohistochemistry among ISH amplified cases [27].

In one previous study on patients with resected pancreatic cancer, high HER3 expression was denoted in 41% of cases, and was found to be associated with a shorter OS [30], which is in line with studies on several other types of adenocarcinoma [31]. However, high HER3 expression has also been found to correlate with a prolonged survival in colorectal cancer [32, 33], breast cancer [34] and in gastric and oesophageal cancer [35]. Anti-HER3 antibodies have been shown to reduce growth in pancreatic cancer cell lines that are wild-type for KRAS [36]. To the best of our knowledge, the expression and prognostic impact of HER3 has not been studied in the full spectrum of periampullary adenocarcinoma.

Thus, the prognostic role of HERs in periampullary adenocarcinoma has mostly been studied in pancreatic cancer, often with conflicting results, and less in I-type adenocarcinomas. The efficacy of treatments targeted at EGFR, HER2 and HER3 are not well studied in periampullary cancer, in particular in I-type tumours. Therefore, the aim of this study was to analyse the expression and prognostic significance of EGFR, HER2 and HER3, with particular reference to morphological subtype, in a retrospective, consecutive cohort of 175 cases with periampullary cancer.

Materials and Methods

Patients

The study cohort is a previously described retrospective consecutive series of pancreaticoduodenectomy specimens from all patients (n = 175) with periampullary adenocarcinoma, including pancreatic cancer, resected at the University hospitals of Lund and Malmö, Sweden, from January 1 2001 until December 31 2011 [37–41]. Data on survival were gathered from the Swedish National Civil Register. Follow-up started at the date of surgery and ended at death, at 5 years after surgery or at December 31 2013, whichever came first. Information on neoadjuvant and adjuvant treatment and recurrence was obtained from patient records. All haematoxylin & eosin stained slides from all cases were re-evaluated by one pathologist (JEL), blinded to the original report and outcome, as previously described, to get a uniform assessment of all histopathological parameters.

All EU and national regulations and requirements for handling human samples have been fully complied with during the conduct of this project; i.e. decision no. 1110/94/EC of the European Parliament and of the Council (OJL126 18,5,94), the Helsinki Declaration on ethical principles for medical research involving human subjects, and the EU Council Convention on human rights and Biomedicine. The study was approved of by the Ethics committee of Lund University (ref no 445/07), whereby the committee waived the need for consent other than by the option to opt out. All information from the patient records was anonymized and de-identified prior to analysis.

Tissue microarray construction

Tissue microarrays (TMAs) were constructed using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminister, MD, USA). A standard set of three tissue cores (1 mm) were obtained from each of the 175 formalin fixated paraffin embedded primary tumours and from lymph node metastases from 105 of the cases, whereby one to three lymph node metastases were sampled in each case.

Immunohistochemistry, Silver In-Situ Hybridization and staining evaluation

All immunohistochemical staining and *in situ* hybridization (ISH) was performed on 4 μm TMA-sections. Immunohistochemistry for EGFR and HER3 was performed in an Autostainer Plus (Dako, Glostrup, Denmark) after automated pre-treatment with the PT-link system (Dako), using the monoclonal anti-EGFR antibody 31G7 (Zymed Laboratories Inc, San Francisco, CA, USA), diluted 1:25 and the monoclonal anti-HER3 antibody SP71 (Novus Biologicals LTD, Cambridge, UK), diluted 1:100, respectively. HER2 immunohistochemistry was performed on a BenchMark ULTRA instrument (Ventana Medical Systems, Inc. Tucson, AZ, USA). ULTRA Cell Conditioning (ULTRA CC1), pH9, was used for heat induced epitope retrieval (HIER). The monoclonal primary antibody PATHWAY anti-HER-2/neu (4B5), (Ventana Medical Systems, Inc.) was incubated for 20 minutes and the antibody-antigen complex was visualized with ultraView Universal DAB Detection kit (Ventana Medical Systems, Inc.)

HER2 ISH was also performed on the BenchMark ULTRA instrument (Ventana Medical Systems, Inc.). The peptide bonds were broken with ULTRA Cell Conditioning (ULTRA CC2), pH6, and ISH protease3. HER2 gene and Chromosome 17 (Chr17) were detected with INFORM HER2 Dual ISH DNA Probe Cocktail Assay (Ventana Medical Systems, Inc.). For visualization, ultraView SISH DNP Detection Kit and ultraView Red ISH DIG Detection Kit (Ventana Medical Systems, Inc.) were used, giving black and red chromogenic signals. As a final step, all slides were counterstained with haematoxylin.

The herein used anti-HER3 antibody has been validated by siRNA-mediated knockdown, immunocytochemistry and quantitative real-time PCR [35].

The immunohistochemical staining of EGFR, HER2 and HER3 was annotated by two independent observers (JEL and MH for EGFR, JEL and CFW for HER2/3) and consensus was reached in discordant cases.

EGFR, HER2 and HER3 protein expression was evaluated using the recommended protocol for HER2 testing in gastric and gastroesophageal junction cancer biopsies [42], taking complete, basolateral, or lateral membranous reactivity in a minimum of 5 clustered positive cancer cells into account, with the intensity recorded as 0, 1+, 2+ or 3+. Cytoplasmic staining was denoted as a separate category, but grouped with 1+ in the statistical analyses. Protein expression was grouped 0–2+ vs 3+, whereby 0–2+ was regarded as low expression and 3+ as high expression.

Assessment of ISH was performed according to the Ventana INFORM HER2 Dual ISH DNA Probe Cocktail Assay Interpretation Guide, and annotated by one pathologist (JEL). For each core 20 cancer cells were counted, and if the resulting HER2/Chr17 ratio fell within 1.5 and 2.5, another 20 cells were counted. A ratio above 2.0 was denoted as amplified. Assessment of HER2 ISH was only performed on cases that had either 2+ or 3+ immunohistochemical HER2 expression.

Statistical analysis

Chi square test was applied to analyse the relationship between the dichotomized expression of each biomarker and clinicopathological parameters. Two patients with PB-type adenocarcinomas who had received neoadjuvant chemotherapy were excluded from the correlation and survival analyses. Three additional patients were excluded from the survival analyses; two with I-type adenocarcinomas who died within one month from surgery due to complications and one with PB-type adenocarcinoma who emigrated 5 months after surgery.

Kaplan Meier estimates of 5-year RFS and OS and log rank test were applied to evaluate survival differences in strata according to high and low expression of each biomarker. For PB-type tumours, biomarker expression was also combined with given adjuvant treatment; gemcitabine vs none/other. To estimate the interaction between given adjuvant treatment and biomarker expression in relation to survival, the following interaction variable was constructed; gemcitabine-based adjuvant treatment (+/−) × biomarker (high/low). Hazard ratios (HR) and 95% confidence intervals (CI) for death and recurrence within 5 years were calculated by Cox regression proportional hazard's modelling in unadjusted analysis and in a multivariable model adjusted for differentiation grade, T-stage, N-stage, perineural invasion, lymphatic invasion, vascular invasion, invasion in peripancreatic fat and adjuvant chemotherapy. A backward conditional method was used for variable selection in the adjusted model. The proportional hazards assumption was tested by examining log-log survival curves.

All tests were two sided. P-values <0.05 were considered significant, and no adjustments were made for the number of tests performed. All statistical analyses were performed using IBM SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Distribution of protein expression of EGFR, HER2 and HER3 and *HER2* gene amplification

Examples of immunohistochemistry scores 0, 1+, 2+ and 3+ for EGFR, HER2 and HER3 expression and *HER2* gene amplification by SISH are shown in Fig 1.

The distribution of expression of EGFR, HER2 and HER3 in PB- and I-type primary tumours is shown in Table 1. For cases with 2+ or 3+ expression of HER2, the results of SISH for *HER2* are also shown. The fraction of cases with 3+ expression differed significantly between PB-type and I-type tumours for HER3 (17% vs 51%, $p<0.001$), and for HER2 (0% vs 6%, $p = 0.017$), but not for EGFR. HER2 2+ expression was seen in 14% of both PB- and I-type tumours. SISH for *HER2* failed in 46% of the cases, probably due to prolonged tissue fixation in formaldehyde. All assessable HER2 3+ cases showed amplification of *HER2*, as did one 2+ PB-type case of ampullary origin. In total, 7% (5/68) of ampullary adenocarcinoma and 8% (4/49) of I-type ampullary adenocarcinoma showed HER2 overexpression (either immunohistochemistry 3+ or SISH+). There were no cases with 3+ co-expression of all three HER family members.

Since there were no tumours with 3+ expression of HER2 in the PB-group, further analyses on associations and prognosis related to 3+ expression of HER2 in PB-type tumours could not be done.

In the full cohort there were 86 cases with evaluable stainings from both primary tumour and corresponding lymph node metastases. There were no significant differences in the proportion of 3+ expression of EGFR, HER2 or HER3 between primary tumours and corresponding metastases (data not shown).

Associations between EGFR, HER2 and HER3 protein expression and clinicopathological parameters

In PB-type adenocarcinomas there were no significant associations between 3+ expression of EGFR or HER3 and clinicopathological parameters (S1 Table).

In I-type adenocarcinomas, high EGFR expression was significantly associated with larger tumour size, but not with any other parameter. HER2 expression was not associated with any parameter. For HER3, there was an inverse association between high protein expression and

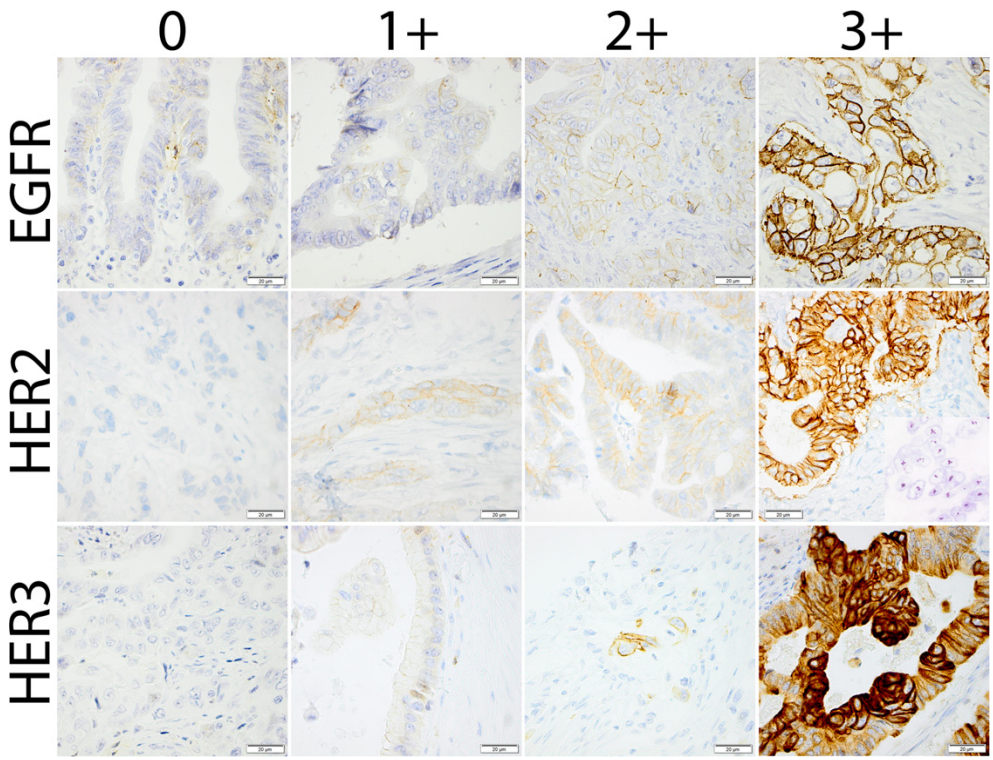


Fig 1. Sample immunohistochemical images. Photomicrographs representing different categories of immunohistochemical staining for EGFR, HER2 and HER3, respectively. An image visualizing *HER2* gene amplification by silver in situ hybridization is shown together with the HER2 3+ case.

doi:10.1371/journal.pone.0153533.g001

tumour stage, perineural growth, blood vessel invasion, growth in peripancreatic fat and recurrence (S2 Table). When considering I-type adenocarcinomas of ampullary origin only, thus excluding duodenal origin, the associations remained significant for tumour stage ($p<0.001$), perineural growth ($p = 0.002$) and growth in peripancreatic fat ($p<0.001$) (data not shown).

Impact of EGFR, HER2 and HER3 expression on 5-year recurrence-free and overall survival

In the full group of PB-type cases, recurrence-free survival (RFS) and OS did not differ by expression of EGFR or HER3 (Fig 2A–2D). Analysis in strata according to adjuvant treatment, however, revealed a significantly reduced RFS and OS for patients that had received adjuvant gemcitabine and had tumours with high, as compared with low, EGFR expression (Fig 3A and 3B), whereas no survival difference was seen according to high or low EGFR expression among

Table 1. Expression of EGFR, HER2 and HER3, and amplification status for *HER2* in pancreatobiliary and intestinal type periampullary adenocarcinoma.

		PB-type	I-type	All
		n = 110	n = 65	n = 175
Excluded due to neoadjuvant treatment		2	0	2
EGFR IHC score				
	0	2 (2%)	2 (3%)	4 (2%)
	1	18 (17%)	16 (25%)	34 (20%)
	2	39 (36%)	22 (35%)	61 (36%)
	3	48 (45%)	23 (37%)	71 (42%)
	Unassessable	1	2	3
HER2 IHC score				
	0	48 (44%)	27 (43%)	75 (44%)
	1	45 (42%)	23 (37%)	68 (40%)
	2	15 (14%)	9 (14%)	24 (14%)
	3	0 (0%)	4 (6%)	4 (2%)
	Unassessable	0	2	2
HER2 SISH (for IHC 2/3+)				
	Not amplified	8 (53%)	4 (31%)	12 (43%)
	Amplified	1 (7%)	2 (15%)	3 (11%)
	Failed SISH	6 (40%)	7 (54%)	13 (46%)
	Not assessed	93	52	145
HER3 IHC score				
	0	67 (63%)	18 (29%)	85 (50%)
	1	18 (17%)	12 (19%)	30 (18%)
	2	4 (4%)	1 (2%)	5 (3%)
	3	18 (17%)	32 (51%)	50 (29%)
	Unassessable	1	2	3

IHC: immunohistochemistry. SISH: silver in situ hybridization. Due to rounding effects, percentages may not add up to 100.

doi:10.1371/journal.pone.0153533.t001

untreated PB-type cases (Fig 3). As further shown in Table 2, there was a significant treatment interaction between EGFR expression and adjuvant gemcitabine in relation to OS ($p_{interaction}$ = 0.042), but not in relation to RFS. When considering only pancreatic tumour origin, a significantly shorter RFS (HR 2.47, 95% CI 1.02–6.00) and OS (HR 3.47, 95% CI 1.34–8.97) was seen in adjuvant gemcitabine treated cases with tumours displaying high EGFR expression, as compared with low EGFR expression, whereas no survival difference was seen according to high or low EGFR expression among cases not receiving adjuvant gemcitabine. There was however no significant interaction in relation to neither RFS, $p(_{interaction})$ = 0.084 nor OS, $p(_{interaction})$ = 0.160.

In I-type cases, Kaplan Meier analysis revealed a significantly shorter OS and RFS for cases with high EGFR expression (Fig 4A and 4B). Significance was retained in univariable Cox regression analysis for RFS (HR 2.58, 95% CI 1.23–5.38) and OS (HR 2.74, 95% CI 1.32–5.69), but not in multivariable analysis (Table 3). The prognostic value of EGFR expression did not differ according to adjuvant treatment in I-type tumours (data not shown), and there was no significant difference in RFS or OS between cases with high or low HER2 expression (Fig 4C and 4D and Table 3). There was a significant association between high HER3 expression and a longer RFS (p = 0.031), and significance was retained in univariable Cox regression analysis (HR 0.45, 95% CI 0.21–0.95), but not in multivariable analysis (Table 3). There was no significant association between HER3 expression and OS.

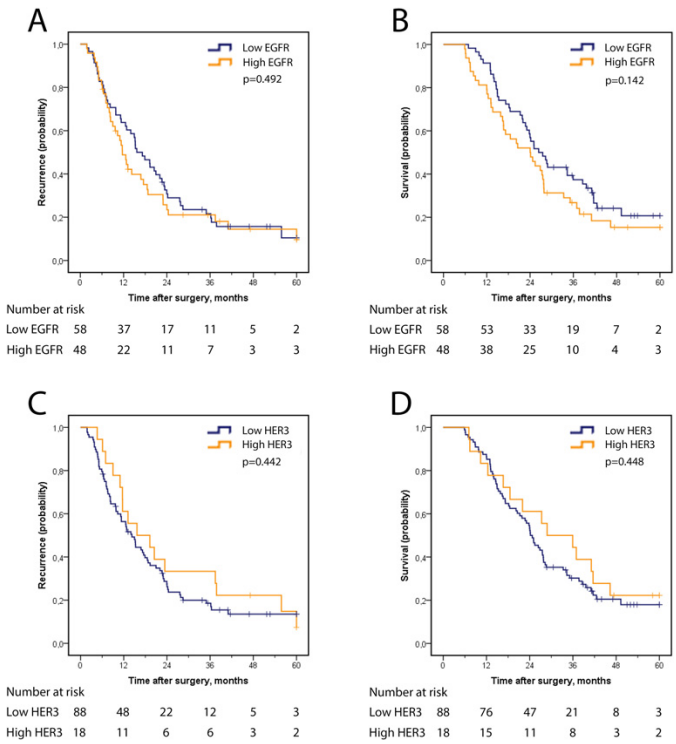


Fig 2. Recurrence-free and overall survival according to EGFR and HER3 expression in PB-type adenocarcinoma. Kaplan Meier analysis of five-year recurrence-free survival in strata according to high and low expression of (A) EGFR and (C) HER3 and overall survival according to high and low expression of (B) EGFR and (D) HER3.

doi:10.1371/journal.pone.0153533.g002

Discussion

In this study, we have evaluated the prognostic impact of EGFR, HER2 and HER3 in periampullary adenocarcinoma, by morphological type and adjuvant treatment. In intestinal-type adenocarcinoma, we found that high HER3 expression was a favourable prognostic factor and that high EGFR expression was an adverse prognostic factor, although none of these associations were independent of other prognostic factors. In addition, in pancreatobiliary-type tumours, EGFR expression was found to be an adverse prognostic factor only in cases that received adjuvant gemcitabine, and a positive effect of gemcitabine was only seen in cases with low EGFR expression, with a significant interaction between EGFR expression and adjuvant gemcitabine in relation to overall survival. HER2 expression was not prognostic, neither in intestinal-type, nor in pancreatobiliary-type tumours.

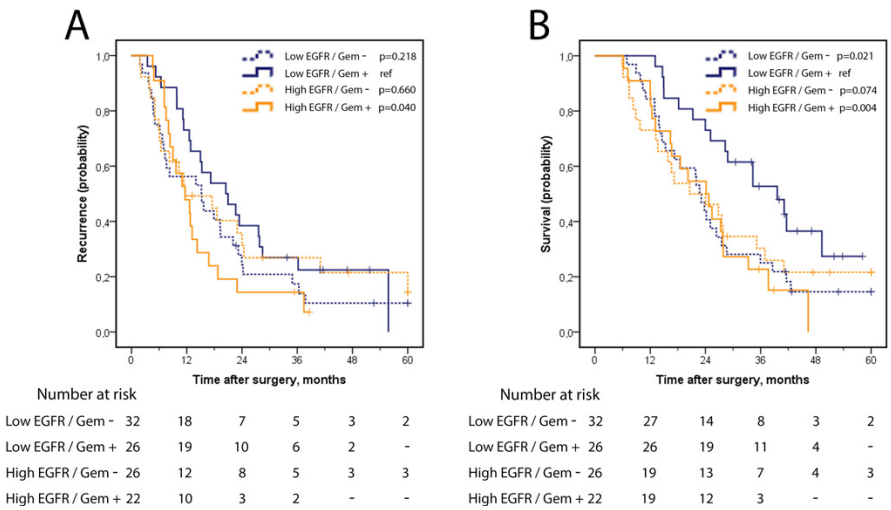


Fig 3. Recurrence-free and overall survival in strata according to EGFR expression and adjuvant gemcitabine in PB-type adenocarcinoma. Kaplan Meier analysis of (A) five-year recurrence-free survival and (B) overall survival in combined strata according to EGFR expression (high/low) and adjuvant gemcitabine (yes/no).

doi:10.1371/journal.pone.0153533.g003

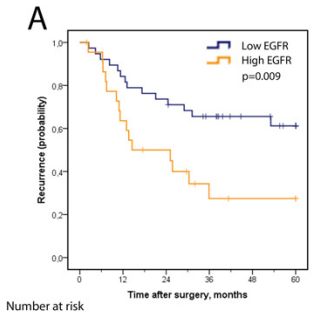
Our results regarding the expression of EGFR, HER2 and HER3 are comparable to previously published results, with the possible exception of EGFR expression in I-type adenocarcinomas, where we found 3+ expression in 38% of the cases, compared with 4% expression [14] and 19% 3+ expression [15] in other studies. In addition, we were not able to demonstrate the previously described association between HER2 and HER3 expression. Our results regarding the adverse prognostic effect of EGFR in the entire group of intestinal-type, but not in pancreatobiliary type, adenocarcinoma confirm the results of Xia et al [15], and further underscore the

Table 2. Cox proportional hazards analysis of the impact of EGFR expression on recurrence-free and overall survival in strata according to adjuvant gemcitabine in patients with PB-type adenocarcinoma.

	RFS		OS	
	HR (95% CI)	**p(interaction)**	**HR (95% CI)**	**p(interaction)**
Gem -				
Low EGFR (n = 32)	1.00		1.00	
High EGFR (n = 26)	0.84 (0.47–1.49)	0.098	0.94 (0.53–1.68)	**0.042**
Gem +				
Low EGFR (n = 26)	1.00		1.00	
High EGFR (n = 22)	**1.93 (1.02–3.65)**		**2.69 (1.34–5.42)**	

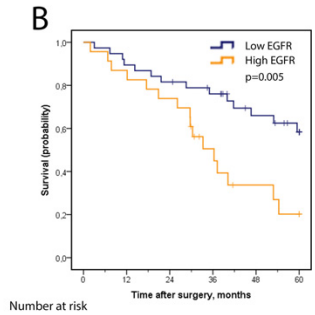
Bold text indicates significant values

doi:10.1371/journal.pone.0153533.t002



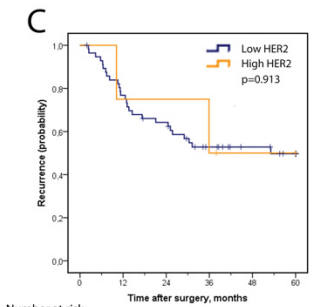
Number at risk

Low EGFR	38	32	28	22	16	12
High EGFR	23	14	10	4	3	3



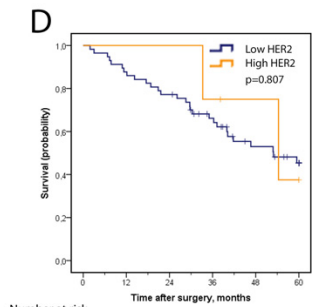
Number at risk

Low EGFR	38	34	31	27	19	14
High EGFR	23	20	17	9	5	3



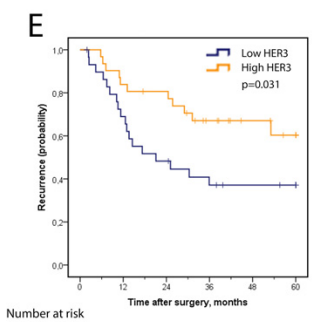
Number at risk

Low HER2	57	43	35	24	18	14
High HER2	4	3	3	2	1	1



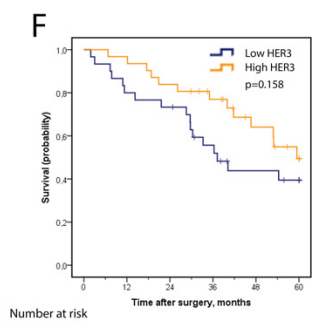
Number at risk

Low HER2	57	50	44	33	22	16
High HER2	4	4	4	3	2	1



Number at risk

Low HER3	30	20	14	10	8	7
High HER3	31	26	24	16	11	8



Number at risk

Low HER3	30	24	20	15	10	8
High HER3	31	30	26	21	14	9

Fig 4. Recurrence-free and overall survival according to EGFR, HER2 and HER3 expression in I-type adenocarcinoma. Kaplan Meier analysis of five-year recurrence-free survival in strata according to high and low expression of (A) EGFR, (C) HER2, (E) HER3 and overall survival according to high and low expression of (B) EGFR, (D) HER2, and (F) HER3.

doi:10.1371/journal.pone.0153533.g004

biological differences between pancreatobiliary and intestinal type periampullary adenocarcinomas. Our finding of a positive effect of gemcitabine only in cases with low EGFR expression has to our knowledge not been shown in these tumour types before. This finding is however compatible with the described increase in EGFR expression in colon cancer cell lines when resistance to chemotherapy was induced [43], and a better response to chemoradiotherapy in esophageal squamous cell carcinoma displaying low EGFR expression [44]. Our finding thus suggests that gemcitabine has a limited or no effect on survival in pancreatobiliary type tumours with high expression of EGFR. From a mechanistic viewpoint, inhibition of EGFR could theoretically seem like an attractive treatment option in these patients. However, immunohistochemical assessment of EGFR expression has not been a good predictor of response to the EGFR antibodies cetuximab or panitumumab in colorectal cancer, compared with *EGFR* copy number and *KRAS* mutation analysis [45–48]. Expression of EGFR also failed to predict response to the EGFR tyrosine kinase inhibitor erlotinib, when added to gemcitabine in the NCIC CTG PA.3 trial, in patients with locally advanced or metastatic pancreatic cancer [13]. Although the herein studied cohort contains both adjuvant treated and untreated patients, thus enabling identification of potential predictive biomarkers, firm conclusions on treatment prediction should not be drawn, given the retrospective character of the cohort. Another caveat is that several tests have been made in the present study, which increases the risk for type I errors, i.e. detecting a difference that is coincidental.

Another possible limitation to the present study is the use of tissue microarrays, whereby the issue of representativity in relation to whole tissue sections may always be raised. One should however bear in mind that whole tissue sections also represent only a minor part of the tumour, and that the tissue microarray technique allows sampling from different regions in different tissue blocks, thus enabling detection of heterogeneous expression. With a few exceptions [49], the tissue microarray method accurately reflects the expression of different proteins, and is a well-validated platform for studies of biomarkers [50].

Table 3. Cox proportional hazards analysis of the impact of EGFR, HER2 and HER3 expression on recurrence-free and overall survival in patients with I-type adenocarcinoma.

	RFS			OS		
	n (events)	Univariable	Multivariable	n (events)	Univariable	Multivariable
EGFR						
Low	38 (14)	1.00	1.00	38 (14)	1.00	1.00
High	23 (15)	**2.58 (1.23–5.38)**	1.80 (0.85–3.82)	23 (16)	**2.74 (1.32–5.69)**	1.55 (0.68–3.53)
HER2						
Low	57 (27)	1.00		57 (28)	1.00	
High	4 (2)	0.92 (0.22–3.89)	NI	4 (2)	0.84 (0.20–3.51)	NI
HER3						
Low	30 (18)	1.00	1.00	30 (17)	1.00	
High	31 (11)	**0.45 (0.21–0.95)**	1.37 (0.52–3.62)	31 (13)	0.60 (0.29–1.23)	NI

Multivariable analysis adjusted for tumour grade, N-stage (N0 vs N1), T-stage (T1-2 vs T3-4), perineural growth, lymphatic invasion, blood vessel invasion, invasion of perineural fat and adjuvant treatment. NI, not investigated. Bold text indicates significant values.

doi:10.1371/journal.pone.0153533.t003

Our results on the incidence of HER2 overexpression (3+ and/or ISH amplification) are well in line with previous results, with a low frequency in pancreatobiliary-type tumours or pancreatic cancer, and a higher frequency in I-type adenocarcinomas, although the latter is lower than in gastric cancer [42]. The HER2-HER3 homodimer is a powerful activator of the PI3K/Akt pathway [51], causing aberrant proliferative and antiapoptotic intracellular signals [52]. Inhibition of HER2 activity, however, causes upregulation of HER3, and simultaneous blockage of HER2 and HER3 activity gives a more potent inhibition of HER2 dependent oncogenic features than blockage of one receptor alone [53]. In line with those findings there is one case report of second line therapy with the antibodies trastuzumab and pertuzumab, thus inhibiting both HER2 and its dimerization, for HER2 overexpressing metastatic ampullary cancer, describing stable disease, with some shrinkage of metastases, and a longer survival than expected upon standard chemotherapy [54]. Blockage of HER3 activity is thus of interest, and clinical trials with the HER3 antibody patritumab are ongoing [55, 56].

Our finding of a longer recurrence-free survival in I-type cases with high HER3 expression is unexpected, given the oncogenic features of HER3, and in contrast with previous reports on various non-gastrointestinal cancers [31], one on colon cancer [57], and two on gastric cancer [58, 59], but harmonize with a few studies on breast [34], colorectal [32, 33], and gastric and oesophageal cancer [35]. A possible explanation for our finding could be that high HER3 expression reflects a less proliferative tumour, which is in line with the described expression of HER3 in non-proliferating parts of colon epithelium and colon cancer [60]. Another explanation for the diverging results regarding the prognostic effect of HER3 could also be the use of different antibodies, and algorithms for assessing the expression. The antibody used in the present study is however well validated [35], and we have used the well-known protocol for assessing HER2-immunohistochemistry in biopsies of gastric cancer, to make the annotation easily reproducible.

In the current study, the least studied HER family member, HER4, was not included, but given the complex network of signalling pathways that combinations of HER dimers and ligands can activate, it is not unlikely that expression of HER4 may have prognostic or predictive implications in periampullary adenocarcinoma.

In summary, the results from the present study demonstrate that high EGFR expression is an unfavourable prognostic factor in in gemcitabine treated pancreatobiliary type adenocarcinoma. The finding of a potential interaction between EGFR expression and response to adjuvant gemcitabine in pancreatobiliary type tumours is novel and of potential clinical relevance, and therefore merits confirmation and further study, both in a mechanistic context as well as in additional patient cohorts. EGFR expression was also an unfavourable prognostic factor, although not independent from other factors, in intestinal type tumours. Expression of HER3 was found to differ between pancreatobiliary and intestinal type adenocarcinomas and to be a favourable prognostic factor, however not independent, in intestinal type adenocarcinoma. Overexpression of HER2 was observed in 8% of intestinal type ampullary adenocarcinoma, and was not associated with prognosis. It is feasible that further steps towards individualized therapy in periampullary adenocarcinoma will involve simultaneous targeting of several members of the HER family.

Supporting Information

S1 Table. Associations between expression of EGFR, HER3 and clinicopathological parameters in pancreatobiliary-type periampullary adenocarcinoma. M, median. IQR, interquartile range.
(DOCX)

S2 Table. Associations between expression of EGFR, HER2, HER3 and clinicopathological parameters in intestinal-type periampullary adenocarcinoma. M, median. IQR, interquartile range. Bold text indicates significant values.
(DOCX)

Acknowledgments

This study was supported by grants from the Swedish Research Council, the Swedish Cancer Society, the Gunnar Nilsson Cancer Foundation, the Mrs Berta Kamprad Foundation, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and University Hospital Research Grants.

Author Contributions

Conceived and designed the experiments: KJ J. Eberhard. Performed the experiments: J. Elebro MH CFW. Analyzed the data: J. Elebro. Contributed reagents/materials/analysis tools: BN. Wrote the paper: J. Elebro MH CFW J. Eberhard KJ.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015; 65(1):5–29. doi: 10.3322/caac.21254 PMID: 25559415.
2. Engholm G, Ferlay J, Christensen N, Kejs AMT, Johannesen TB, Khan S, et al. NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 7.1 (09.07.2015).: Association of the Nordic Cancer Registries. Danish Cancer Society.; 2015. Available: http://www.ancr.nu.
3. Carpelan-Holmstrom M, Nordling S, Pukkala E, Sankila R, Luttges J, Kloppel G, et al. Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry. Gut. 2005; 54(3):385–7. doi: 10.1136/gut.2004.047191 PMID: 15710987; PubMed Central PMCID: PMC1774412.
4. Westgaard A, Tafjord S, Farstad IN, Cvancarova M, Eide TJ, Mathisen O, et al. Pancreatobiliary versus intestinal histologic type of differentiation is an independent prognostic factor in resected periampullary adenocarcinoma. BMC Cancer. 2008; 8:170. doi: 10.1186/1471-2407-8-170 PMID: 18547417; PubMed Central PMCID: PMC2430209.
5. Westgaard A, Pomianowska E, Clausen OP, Gladhaug IP. Intestinal-type and pancreatobiliary-type adenocarcinomas: how does ampullary carcinoma differ from other periampullary malignancies? Ann Surg Oncol. 2013; 20(2):430–9. doi: 10.1245/s10434-012-2603-0 PMID: 22956064.
6. Bronsert P, Kohler I, Werner M, Makowiec F, Kuesters S, Hoeppner J, et al. Intestinal-type of differentiation predicts favourable overall survival: confirmatory clinicopathological analysis of 198 periampullary adenocarcinomas of pancreatic, biliary, ampullary and duodenal origin. BMC Cancer. 2013; 13:428. doi: 10.1186/1471-2407-13-428 PMID: 24053229; PubMed Central PMCID: PMCPMC3849372.
7. Roskoski R Jr. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol Res. 2014; 79:34–74. doi: 10.1016/j.phrs.2013.11.002 PMID: 24269963.
8. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015; 372(8):724–34. doi: 10.1056/NEJMoa1413513 PMID: 25693012.
9. Tobita K, Kijima H, Dowaki S, Kashiwagi H, Ohtani Y, Oida Y, et al. Epidermal growth factor receptor expression in human pancreatic cancer: Significance for liver metastasis. Int J Mol Med. 2003; 11(3):305–9. PMID: 12579331.
10. Uegaki K, Nio Y, Inoue Y, Minari Y, Sato Y, Song MM, et al. Clinicopathological significance of epidermal growth factor and its receptor in human pancreatic cancer. Anticancer Res. 1997; 17(5B):3841–7. PMID: 9427790.
11. Bloomston M, Bhardwaj A, Ellison EC, Frankel WL. Epidermal growth factor receptor expression in pancreatic carcinoma using tissue microarray technique. Dig Surg. 2006; 23(1–2):74–9. doi: 10.1159/000093497 PMID: 16717472.
12. Luo G, Long J, Qiu L, Liu C, Xu J, Yu X. Role of epidermal growth factor receptor expression on patient survival in pancreatic cancer: a meta-analysis. Pancreatology. 2011; 11(6):595–600. doi: 10.1159/000334465 PMID: 22261712.

13. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol. 2007; 25(15):1960–6. doi: 10.1200/JCO.2006.07.9525 PMID: 17452677.

14. Mikhitarian K, Pollen M, Zhao Z, Shyr Y, Merchant NB, Parikh A, et al. Epidermal growth factor receptor signaling pathway is frequently altered in ampullary carcinoma at protein and genetic levels. Mod Pathol. 2014; 27(5):665–74. doi: 10.1038/modpathol.2013.185 PMID: 24186143; PubMed Central PMCID: PMCPMC4007414.

15. Xia M, Overman MJ, Rashid A, Chatterjee D, Wang H, Katz MH, et al. Expression and clinical significance of epidermal growth factor receptor and insulin-like growth factor receptor 1 in patients with ampullary adenocarcinoma. Hum Pathol. 2015; 46(9):1315–22. doi: 10.1016/j.humpath.2015.05.012 PMID: 26165226.

16. Aloysius MM, Lobo DN, Rowlands BJ, Madhusudan S, Ilyas M, Zaitoun AM. HER-2/Neu overexpression is a rare event in peri-ampullary cancer: assessment using the HercepTest. Histopathology. 2009; 55(2):236–7. doi: 10.1111/j.1365-2559.2009.03351.x PMID: 19694833.

17. Koeppen HK, Wright BD, Burt AD, Quirke P, McNicol AM, Dybdal NO, et al. Overexpression of HER2/neu in solid tumours: an immunohistochemical survey. Histopathology. 2001; 38(2):96–104. PMID: 11207822.

18. Safran H, Steinhoff M, Mangray S, Rathore R, King TC, Chai L, et al. Overexpression of the HER-2/neu oncogene in pancreatic adenocarcinoma. Am J Clin Oncol. 2001; 24(5):496–9. PMID: 11586103.

19. Chou A, Waddell N, Cowley MJ, Gill AJ, Chang DK, Patch AM, et al. Clinical and molecular characterization of HER2 amplified-pancreatic cancer. Genome Med. 2013; 5(8):78. doi: 10.1186/gm482 PMID: 24004612; PubMed Central PMCID: PMC3978667.

20. Potti A, Ganti AK, Tendulkar K, Chitajallu S, Sholes K, Koch M, et al. HER-2/neu and CD117 (C-kit) overexpression in hepatocellular and pancreatic carcinoma. Anticancer Res. 2003; 23(3B):2671–4. PMID: 12894556.

21. Stoecklein NH, Luebke AM, Erbersdobler A, Knoefel WT, Schraut W, Verde PE, et al. Copy number of chromosome 17 but not HER2 amplification predicts clinical outcome of patients with pancreatic ductal adenocarcinoma. J Clin Oncol. 2004; 22(23):4737–45. doi: 10.1200/JCO.2004.05.142 PMID: 15570074.

22. Day JD, Digiuseppe JA, Yeo C, Lai-Goldman M, Anderson SM, Goodman SN, et al. Immunohistochemical evaluation of HER-2/neu expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasms. Hum Pathol. 1996; 27(2):119–24. PMID: 8617452.

23. Ata A, Polat A, Serinsoz E, Sungur MA, Arican A. Prognostic value of increased HER2 expression in cancers of pancreas and biliary tree. Pathol Oncol Res. 2015; 21(3):831–8. doi: 10.1007/s12253-014-9847-x PMID: 25416598.

24. Komoto M, Nakata B, Amano R, Yamada N, Yashiro M, Ohira M, et al. HER2 overexpression correlates with survival after curative resection of pancreatic cancer. Cancer Sci. 2009; 100(7):1243–7. doi: 10.1111/j.1349-7006.2009.01176.x PMID: 19432892.

25. Koka V, Potti A, Koch M, Fraiman G, Mehdi S, Levitt R. Role of immunohistochemical identification of Her-2/neu and detection of variability in overexpression in pancreatic carcinoma. Anticancer Res. 2002; 22(3):1593–7. PMID: 12168842.

26. Safran H, Iannitti D, Ramanathan R, Schwartz JD, Steinhoff M, Nauman C, et al. Herceptin and gemcitabine for metastatic pancreatic cancers that overexpress HER-2/neu. Cancer Invest. 2004; 22(5):706–12. PMID: 15581051.

27. Hechtman JF, Liu W, Sadowska J, Zhen L, Borsu L, Arcila ME, et al. Sequencing of 279 cancer genes in ampullary carcinoma reveals trends relating to histologic subtypes and frequent amplification and overexpression of ERBB2 (HER2). Mod Pathol. 2015; 28(8):1123–9. doi: 10.1038/modpathol.2015.57 PMID: 25975284.

28. Baumhoer D, Zlobec I, Tornillo L, Dietmaier W, Wuensch PH, Hartmann A, et al. Immunophenotyping and oncogene amplifications in tumors of the papilla of Vater. Virchows Arch. 2008; 453(6):579–88. doi: 10.1007/s00428-008-0669-7 PMID: 18936968.

29. Kawamoto T, Ishige K, Thomas M, Yamashita-Kashima Y, Shu S, Ishikura N, et al. Overexpression and gene amplification of EGFR, HER2, and HER3 in biliary tract carcinomas, and the possibility for therapy with the HER2-targeting antibody pertuzumab. J Gastroenterol. 2015; 50(4):467–79. doi: 10.1007/s00535-014-0984-5 PMID: 25112701.

30. Hirakawa T, Nakata B, Amano R, Kimura K, Shimizu S, Ohira G, et al. HER3 overexpression as an independent indicator of poor prognosis for patients with curatively resected pancreatic cancer. Oncology. 2011; 81(3–4):192–8. doi: 10.1159/000333825 PMID: 22067729.

31. Ocana A, Vera-Badillo F, Seruga B, Templeton A, Pandiella A, Amir E. HER3 overexpression and survival in solid tumors: a meta-analysis. J Natl Cancer Inst. 2013; 105(4):266–73. doi: 10.1093/jnci/djs501 PMID: 23221996.

32. Baiocchi G, Lopes A, Coudry RA, Rossi BM, Soares FA, Aguiar S, et al. ErbB family immunohistochemical expression in colorectal cancer patients with higher risk of recurrence after radical surgery. Int J Colorectal Dis. 2009; 24(9):1059–68. doi: 10.1007/s00384-009-0702-6 PMID: 19390858.

33. Seo AN, Kwak Y, Kim WH, Kim DW, Kang SB, Choe G, et al. HER3 protein expression in relation to HER2 positivity in patients with primary colorectal cancer: clinical relevance and prognostic value. Virchows Arch. 2015; 466(6):645–54. doi: 10.1007/s00428-015-1747-2 PMID: 25739551.

34. Quinn CM, Ostrowski JL, Lane SA, Loney DP, Teasdale J, Benson FA. c-erbB-3 protein expression in human breast cancer: comparison with other tumour variables and survival. Histopathology. 1994; 25 (3):247–52. PMID: 7821892.

35. Hedner C, Borg D, Nodin B, Karnevi E, Jirstrom K, Eberhard J. Expression and Prognostic Significance of Human Epidermal Growth Factor Receptors 1 and 3 in Gastric and Esophageal Adenocarcinoma. PLoS One. 2016; 11(2):e0148101. doi: 10.1371/journal.pone.0148101 PMID: 26844548.

36. Lazrek Y, Dubreuil O, Garambois V, Gaborit N, Larbouret C, Le Clorennec C, et al. Anti-HER3 domain 1 and 3 antibodies reduce tumor growth by hindering HER2/HER3 dimerization and AKT-induced MDM2, XIAP, and FoxO1 phosphorylation. Neoplasia. 2013; 15(3):335–47. PMID: 23479511; PubMed Central PMCID: PMC3593156.

37. Elebro J, Jirstrom K. Use of a standardized diagnostic approach improves the prognostic information of histopathologic factors in pancreatic and periampullary adenocarcinoma. Diagn Pathol. 2014; 9(1):80. doi: 10.1186/1746-1596-9-80 PMID: 24731283.

38. Elebro J, Heby M, Gaber A, Nodin B, Jonsson L, Fristedt R, et al. Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. J Transl Med. 2014; 12(1):289. doi: 10.1186/s12967-014-0289-8

39. Elebro J, Ben Dror L, Heby M, Nodin B, Jirstrom K, Eberhard J. Prognostic effect of hENT1, dCK and HuR expression by morphological type in periampullary adenocarcinoma, including pancreatic cancer. Acta Oncol. 2015:1–11.

40. Fristedt R, Elebro J, Gaber A, Jonsson L, Heby M, Yudina Y, et al. Reduced expression of the polymeric immunoglobulin receptor in pancreatic and periampullary adenocarcinoma signifies tumour progression and poor prognosis. PLoS One. 2014; 9(11):e112728. doi: 10.1371/journal.pone.0112728 PMID: 25397670; PubMed Central PMCID: PMC4232506.

41. Heby M, Elebro J, Nodin B, Jirstrom K, Eberhard J. Prognostic and predictive significance of podocalyxin-like protein expression in pancreatic and periampullary adenocarcinoma. BMC Clin Pathol. 2015; 15:10. doi: 10.1186/s12907-015-0009-1 PMID: 26028992; PubMed Central PMCID: PMC4449563.

42. Ruschoff J, Hanna W, Bilous M, Hofmann M, Osamura RY, Penault-Llorca F, et al. HER2 testing in gastric cancer: a practical approach. Mod Pathol. 2012; 25(5):637–50. doi: 10.1038/modpathol.2011.198 PMID: 22222640.

43. Ekblad L, Johnsson A. Cetuximab sensitivity associated with oxaliplatin resistance in colorectal cancer. Anticancer Res. 2012; 32(3):783–6. PMID: 22399593.

44. Hickey K, Grehan D, Reid IM, O'Briain S, Walsh TN, Hennessy TP. Expression of epidermal growth factor receptor and proliferating cell nuclear antigen predicts response of esophageal squamous cell carcinoma to chemoradiotherapy. Cancer. 1994; 74(6):1693–8. PMID: 7915963.

45. Cappuzzo F, Finocchiaro G, Rossi E, Janne PA, Carnaghi C, Calandri C, et al. EGFR FISH assay predicts for response to cetuximab in chemotherapy refractory colorectal cancer patients. Ann Oncol. 2008; 19(4):717–23. doi: 10.1093/annonc/mdm492 PMID: 17974556.

46. Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, et al. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. Lancet Oncol. 2005; 6(5):279–86. doi: 10.1016/S1470-2045(05)70102-9 PMID: 15863375.

47. Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol. 2005; 23(9):1803–10. doi: 10.1200/JCO.2005.08.037 PMID: 15677699.

48. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 2006; 66(8):3992–5. doi: 10.1158/0008-5472.CAN-06-0191 PMID: 16618717.

49. Linderoth J, Ehinger M, Akerman M, Cavallin-Stahl E, Enblad G, Erlanson M, et al. Tissue microarray is inappropriate for analysis of BCL6 expression in diffuse large B-cell lymphoma. Eur J Haematol. 2007; 79(2):146–9. doi: 10.1111/j.1600-0609.2007.00892.x PMID: 17635238.

50. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, et al. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol. 2001; 159(6):2249–56. doi: 10.1016/S0002-9440(10)63075-1 PMID: 11733374; PubMed Central PMCID: PMC1850582.

51. Soltoff SP, Carraway KL 3rd, Prigent SA, Gullick WG, Cantley LC. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol. 1994; 14(6):3550–8. PMID: 7515147; PubMed Central PMCID: PMCPMC358722.

52. Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia. 2003; 17(3):590–603. doi: 10.1038/sj.leu.2402824 PMID: 12646949.

53. Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, et al. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. Proc Natl Acad Sci U S A. 2011; 108(12):5021–6. doi: 10.1073/pnas.1016140108 PMID: 21385943; PubMed Central PMCID: PMCPMC3064360.

54. O'Hayer K, Farber J, Yeo CJ, Sama AR. HER-2-Positive Ampullary Adenocarcinoma: A Case Report. Case Reports in Pancreatic Cancer. 2015; 1(1):7–10. doi: 10.1089/crpc.2015.29004.koh

55. LoRusso P, Janne PA, Oliveira M, Rizvi N, Malburg L, Keedy V, et al. Phase I study of U3-1287, a fully human anti-HER3 monoclonal antibody, in patients with advanced solid tumors. Clin Cancer Res. 2013; 19(11):3078–87. doi: 10.1158/1078-0432.CCR-12-3051 PMID: 23591447.

56. Nishio M, Horiike A, Murakami H, Yamamoto N, Kaneda H, Nakagawa K, et al. Phase I study of the HER3-targeted antibody patritumab (U3-1287) combined with erlotinib in Japanese patients with non-small cell lung cancer. Lung Cancer. 2015; 88(3):275–81. doi: 10.1016/j.lungcan.2015.03.010 PMID: 25891541.

57. Beji A, Horst D, Engel J, Kirchner T, Ullrich A. Toward the prognostic significance and therapeutic potential of HER3 receptor tyrosine kinase in human colon cancer. Clin Cancer Res. 2012; 18(4):956–68. doi: 10.1158/1078-0432.CCR-11-1186 PMID: 22142822.

58. Hayashi M, Inokuchi M, Takagi Y, Yamada H, Kojima K, Kumagai J, et al. High expression of HER3 is associated with a decreased survival in gastric cancer. Clin Cancer Res. 2008; 14(23):7843–9. doi: 10.1158/1078-0432.CCR-08-1064 PMID: 19047113.

59. Begnami MD, Fukuda E, Fregnani JH, Nonogaki S, Montagnini AL, da Costa WL Jr., et al. Prognostic implications of altered human epidermal growth factor receptors (HERs) in gastric carcinomas: HER2 and HER3 are predictors of poor outcome. J Clin Oncol. 2011; 29(22):3030–6. doi: 10.1200/JCO.2010.33.6313 PMID: 21709195.

60. Jarde T, Kass L, Staples M, Lescesen H, Carne P, Oliva K, et al. ERBB3 Positively Correlates with Intestinal Stem Cell Markers but Marks a Distinct Non Proliferative Cell Population in Colorectal Cancer. PLoS One. 2015; 10(9):e0138336. doi: 10.1371/journal.pone.0138336 PMID: 26367378; PubMed Central PMCID: PMCPMC4569358.

S1 Table. Associations between expression of EGFR, HER3 and clinicopathological parameters in pancreatobiliary-type periampullary adenocarcinoma.

	EGFR			HER3		
	low, 0-2+ (n=60)	high, 3+ (n=49)	p-value	low, 0-2+ (n=90)	high, 3+ (n=19)	p-value
Excluded, neoadjuvant treatment	1	1		1	1	
Lost to follow up	1			1		
EGFR						0.194
low				51 (88%)	7 (12%)	
high				37 (77%)	11 (23%)	
HER3			0.194			
low	51 (58%)	37 (42%)				
high	7 (39%)	11 (61%)				
Year of surgery, M (IQR)	2009 (2005-2010)	2009 (2005-2010)	0.206	2009 (2006-2010)	2007 (2005-2010)	0.329
Age, M (IQR)	67 (62-73)	67 (61-73)	0.615	66 (61-73)	70 (65-74)	0.402
Sex			0.118			1.000
Women	23 (46%)	27 (54%)		42 (84%)	8 (16%)	
Men	35 (62%)	21 (38%)		46 (82%)	10 (18%)	
Tumour origin			0.058			0.055
Ampulla Vateri	15 (79%)	4 (21%)		19 (100%)	0 (0%)	
Distal bile duct	21 (48%)	23 (52%)		36 (82%)	8 (18%)	
Pancreas	22 (51%)	21 (49%)		33 (77%)	10 (23%)	
Tumour size, mm, M (IQR)	30 (25-35)	30 (23-35)	0.992	30 (25-35)	30 (21-40)	0.068
Differentiation grade			0.071			0.593
Well / moderate	26 (67%)	13 (33%)		31 (79%)	8 (21%)	
Poor	32 (48%)	35 (52%)		57 (85%)	10 (15%)	
T-stage			0.218			0.119
T1 / T2	9 (75%)	3 (25%)		8 (67%)	4 (33%)	
T3 / T4	49 (52%)	45 (48%)		80 (85%)	14 (15%)	
N-stage			0.827			0.087
N0	15 (52%)	14 (48%)		21 (72%)	8 (28%)	
N1	43 (56%)	34 (44%)		67 (87%)	10 (13%)	
Perineural growth			0.638			0.054
No	11 (50%)	11 (50%)		15 (68%)	7 (32%)	
Yes	47 (56%)	37 (44%)		73 (87%)	11 (13%)	
Growth in lymphatic vessels			0.835			0.782
No	17 (53%)	15 (47%)		26 (81%)	6 (19%)	
Yes	41 (55%)	33 (45%)		62 (84%)	12 (16%)	
Growth in blood vessels			0.306			0.413
No	41 (59%)	29 (41%)		60 (86%)	10 (14%)	
Yes	17 (47%)	19 (53%)		28 (78%)	8 (22%)	
Growth in peripancreatic fat			0.810			0.523
No	13 (59%)	9 (41%)		17 (77%)	5 (23%)	
Yes	45 (54%)	39 (46%)		71 (85%)	13 (15%)	
Margins			0.687			0.269

R0	4 (67%)	2 (33%)		4 (67%)	2 (33%)	
R1/Rx	54 (54%)	46 (46%)		84 (84%)	16 (16%)	
Adjuvant treatment			1.000			0.796
No gemcitabine	32 (55%)	26 (45%)		49 (84%)	9 (16%)	
Gemcitabine	26 (54%)	22 (46%)		39 (81%)	9 (19%)	
Recurrence			0.929			0.636
None	9 (50%)	9 (50%)		16 (89%)	2 (11%)	
Local	16 (55%)	13 (45%)		25 (86%)	4 (14%)	
Distant	33 (56%)	26 (44%)		47 (80%)	12 (20%)	

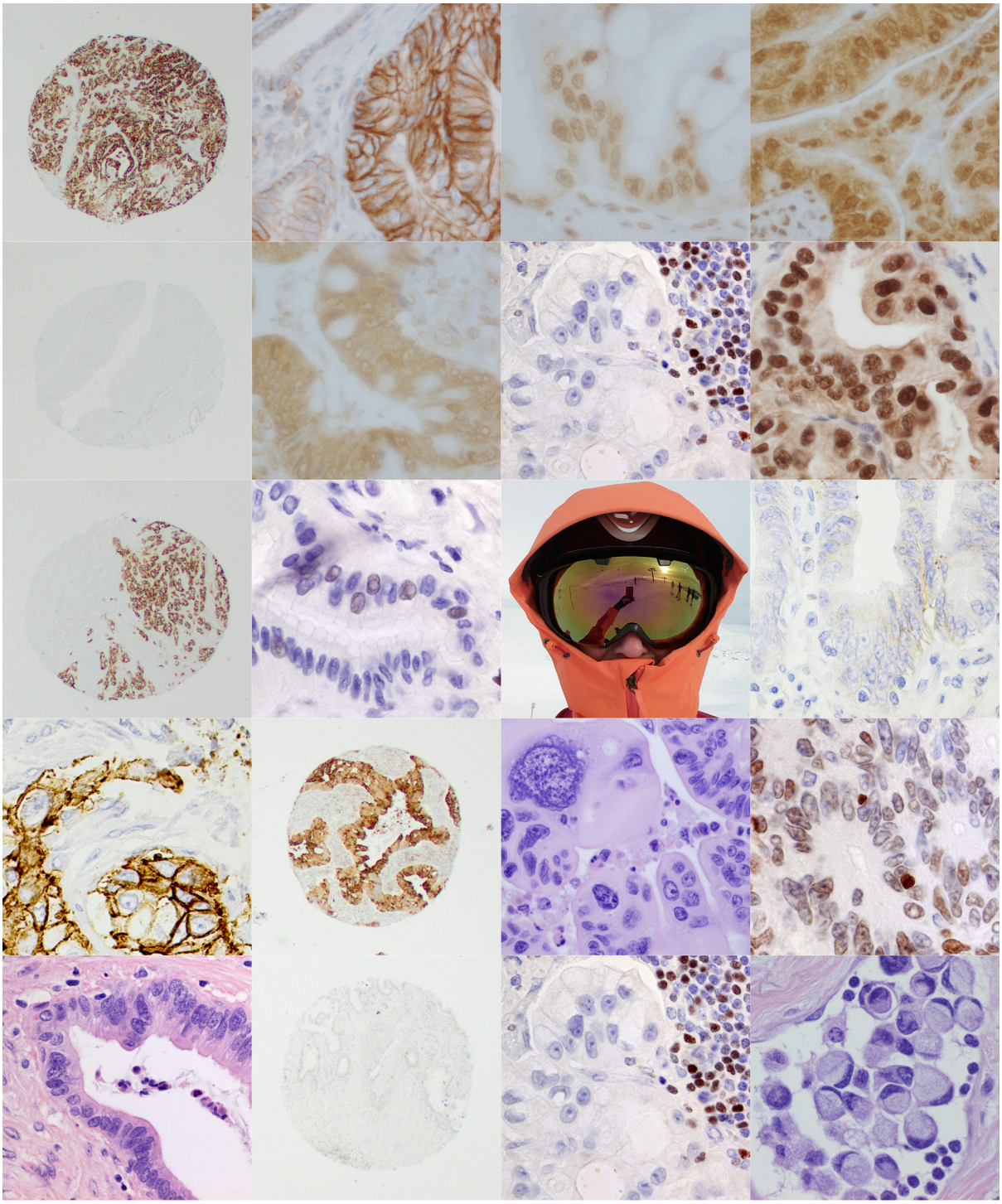
M, median. IQR, interquartile range.

S2 Table. Associations between expression of EGFR, HER2, HER3 and clinicopathological parameters in intestinal-type periampullary adenocarcinoma.

	EGFR			HER2			HER3		
	low, 0-2+ (n=40)	high, 3+ (n=23)	p-value	low, 0-2+ (n=59)	high, 3+ (n=4)	p-value	low, 0-2+ (n=31)	high, 3+ (n=32)	p-value
Excluded, death within 1 month	2	0		2	0		1	1	
Lost to follow up	0	0		0	0		0	0	
EGFR						0.628			0.192
low				36 (95%)	2 (5%)		16 (42%)	22 (58%)	
high				21 (91%)	2 (9%)		14 (61%)	9 (39%)	
HER2			0.628						0.354
low	36 (63%)	21 (37%)					27 (47%)	30 (53%)	
high	2 (50%)	2 (50%)					3 (75%)	1 (25%)	
HER3			0.192			0.354			
low	16 (53%)	14 (47%)		27 (90%)	3 (10%)				
high	22 (71%)	9 (29%)		30 (97%)	1 (3%)				
Year of surgery, M (IQR)	2006 (2003-2009)	2008 (2005-2010)	0.562	2007 (2004-2010)	2007 (2002-2010)	0.292	2006 (2003-2009)	2007 (2005-2010)	0.670
Age, M (IQR)	67 (59-71)	66 (62-69)	0.754	66 (60-70)	56 (46-74)	0.295	65 (58-69)	68 (60-71)	0.885
Sex			1.000			1.000			0.799
Women	21 (62%)	13 (38%)		32 (94%)	2 (6%)		16 (47%)	18 (53%)	
Men	17 (63%)	10 (37%)		25 (93%)	2 (7%)		14 (52%)	13 (48%)	
Tumour origin			1.000			0.569			0.534
Duodenum	8 (62%)	5 (38%)		13 (100%)	0 (0%)		5 (38%)	8 (62%)	
Ampulla, intestinal type	30 (62%)	18 (38%)		44 (92%)	4 (8%)		25 (52%)	23 (48%)	
Tumour size, mm, M (IQR)	25 (15-40)	30 (15-40)	**0.020**	30 (15-40)	23 (16-29)	0.946	30 (23-40)	20 (13-40)	0.118
Differentiation grade			0.114			0.612			0.204
Well / moderate	22 (73%)	8 (27%)		29 (97%)	1 (3%)		12 (40%)	18 (60%)	
Poor	16 (52%)	15 (48%)		28 (90%)	3 (10%)		18 (58%)	13 (42%)	
T-stage			0.214			0.565			**0.005**
T1 / T2	11 (79%)	3 (21%)		14 (100%)	0 (0%)		2 (14%)	12 (86%)	
T3 / T4	27 (57%)	20 (43%)		43 (91%)	4 (9%)		28 (60%)	19 (40%)	
N-stage			0.440			0.618			0.309
N0	19 (58%)	14 (42%)		30 (91%)	3 (9%)		14 (42%)	19 (58%)	
N1/N2	19 (68%)	9 (32%)		27 (96%)	1 (4%)		16 (57%)	12 (43%)	
Perineural growth			0.394			0.582			**0.013**
No	28 (67%)	14 (33%)		40 (95%)	2 (5%)		16 (38%)	26 (62%)	
Yes	10 (53%)	9 (47%)		17 (89%)	2 (11)		14 (74%)	5 (26%)	
Growth in lymphatic vessels			0.440			0.618			0.444
No	19 (68%)	9 (32%)		27 (96%)	1 (4%)		12 (43%)	16 (57%)	
Yes	19 (58%)	14 (42%)		30 (91%)	3 (9%)		18 (55%)	15 (45%)	
Growth in blood			1.000			1.000			**0.024**

vessels									
No	35 (62%)	21 (38%)		52 (93%)	4 (7%)		25 (45%)	31 (55%)	
Yes	3 (60%)	2 (40%)		5 (100%)	0 (0%)		5 (100%)	0 (0%)	
Growth in peripancreatic fat			0.103			0.602			**<0.001**
No	28 (70%)	12 (30%)		38 (95%)	2 (5%)		13 (33%)	27 (67%)	
Yes	10 (48%)	11 (52%)		19 (90%)	2 (10%)		17 (81%)	4 (19%)	
Margins			0.075			1.000			1.000
R0	14 (82%)	3 (18%)		16 (94%)	1 (6%)		8 (47%)	9 (53%)	
R1/Rx	24 (55%)	20 (45%)		41 (93%)	3 (7%)		22 (50%)	22 (50%)	
Adjuvant treatment			0.775			1.000			0.270
No adjuvant	26 (60%)	17 (40%)		40 (93%)	3 (7%)		19 (44%)	24 (56%)	
Any adjuvant	12 (67%)	6 (33%)		17 (94%)	1 (6%)		11 (61%)	7 (39%)	
Recurrence			0.073			1.000			**0.036**
None	24 (75%)	8 (25%)		30 (94%)	2 (6%)		12 (38%)	20 (62%)	
Local	2 (50%)	2 (50%)		4 (100%)	0 (0%)		1 (25%)	3 (75%)	
Distant	12 (48%)	13 (52%)		23 (92%)	2 (8%)		17 (68%)	8 (32%)	

M, median. IQR, interquartile range. Bold text indicates significant values.



LUND UNIVERSITY
Faculty of Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2016:48
ISBN 978-91-7619-274-0
ISSN 1652-8220