Classification and Treatment of Pancreatic and Non-Pancreatic Periampullary Cancers

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Classification and Treatment of Pancreatic and Non-Pancreatic Periampullary Cancers

Classificatie en behandeling van pancreas en periampullaire carcinomen
die niet van het exocriene pancreas uitgaan

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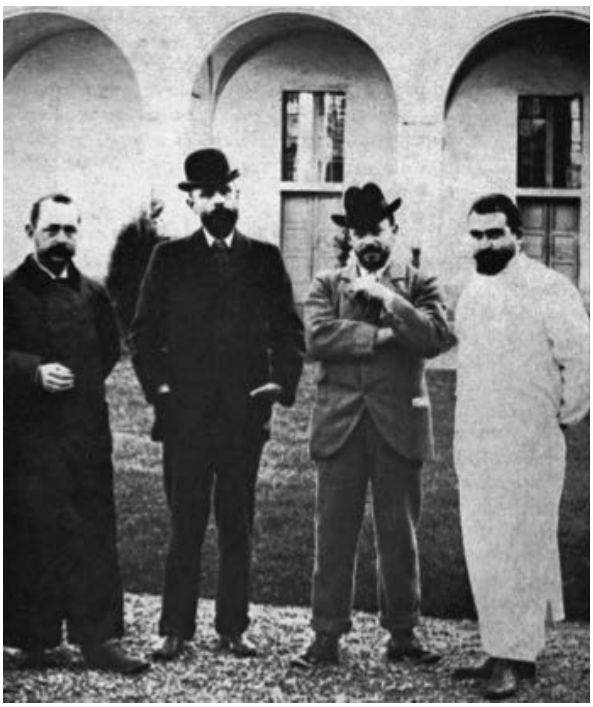
Chapter 1

General Introduction and Outline of the Thesis

Pancreatic cancer and surgery

Since John Baptist Morgagni first described pancreatic cancer in the 18th century, it has been a challenge to treat this devastating disease. Before the end of the 19th century, pancreatic surgery was rarely attempted. It was in 1898 that Allesandro Codivilla, an orthopaedic surgeon[1], made a first attempt to perform a 1-stage pancreaticoduodenectomy[2]. The pancreatic remnant was left intra-abdominally without enterostomy. The patient died 21 days postoperatively, with a “serous appearing fluid” leaking continuously from the wound. In 1903, Emil Theodor Kocher introduced his technique of duodenal mobilization, making pancreatic exposure easier. A few years later, in 1909, the first pancreaticoduodenectomy with preservation of the pancreatic remnant, by performing a pancreaticoduodenostomy was performed by Walter Kausch[3].



Picture: 1895/1896 Allessandro Codivilla third from the left in front of his hospital in Imola. (municipal library of Imola, with permission)

Later on, Whipple, Parsons, and Mullins further developed this technique now known as the “standard Whipple resection” and published their results in 1935[4]. Since then, numerous changes were introduced in the technique of pancreaticoduodenectomy, many of them by Whipple himself. For instance, by using silk instead of catgut, the problem of dissolving sutures because of digestive enzymes was overcome. In later years Traverso and Longmire[5] along with Beger[6] popularized the pylorus-preserving pancreaticoduodenectomy (PPPD), first performed by Watson in 1944. Furthermore, the introduction of vitamin K made it possible to perform a 1-stage procedure in jaundiced patients. In 1973, Fortner[7] described a “regional pancreatectomy” in a search for more radical resection, in an attempt to achieve higher cure rates and improved survival. [3,8]

In early series published in the late 1960s postoperative morbidity rates exceeding 60% and mortality rates approaching 25% were reported. The wimproved outcomes and nowadays high-volume institutions report mortality rates of less than 5%, with morbidity remaining high at 30%–60%[9]. The majority of perioperative complications are not life threatening, though they are responsible for increased length of hospital stay and cost, readmission for care, and delays in adjuvant therapy.

The burden of pancreatic cancer

Despite the advances made pancreatic surgery, in the Western world, pancreatic cancer is still a very lethal disease, and ranks fourth in causes of cancer related death. The incidence op pancreatic and hepatobiliary cancer will probably increase further during the next decades and will become second and third leading cause of cancer related death[10].

Unfortunately, only 10 to 20% of all patients are amenable to resection with curative intent. Even after radical resection, survival rates remain low, and long-time survivors are sparse[11-13]. Median survival after radical resection is 18

months; five year survival rate is 10%. After resection of pancreatic cancer, locoregional recurrence is observed frequently. However, most patients will succumb because of early development of distant metastasis. In fact, most patients have micrometastases already at the time of resection. Therefore, pancreatic cancer should be considered a systemic disease and resection should only be performed in combination with (neo)adjuvant treatment[14]. For unresectable locally advanced pancreatic cancer median survival is approximately 6 to 9 months and for metastatic disease 3 to 6 months. In slight contrast, distal bile duct cancer, also located in the pancreatic head and often misclassified as pancreatic cancer bears a much better prognosis, five-year survival rate may be up to 40-50% after resection.

Adjuvant treatment

Although the benefit of adjuvant gemcitabine based chemotherapy for resected pancreatic ductal adenocarcinoma is small in terms of increased survival, an increased cure rate (from 10 to 20% at 5 years) was demonstrated in a randomised trial[15]. In recent years major advances have been made in the palliative medical treatment of pancreatic cancer by introduction of the FOLFIRINOX regimen[16]. Currently, ongoing trails are investigating the effectiveness of FOLFIRINOX in a (neo-) adjuvant setting.

For non-pancreatic periampullary cancers (NPPC), originating from the distal bile duct, duodenum, ampulla and the papilla of Vater, the role of adjuvant therapy remains largely unclear[17]. In the well-known ESPAC-3 “Periampullary Cancer” trial[18], the investigators were unable to demonstrate a survival benefit of adjuvant gemcitabine based chemotherapy for NPPC. In multivariate analysis, however, a modest benefit was found for adjuvant gemcitabine based therapy. In addition, the role of adjuvant radiotherapy for both pancreatic and NPPC appears to be of very limited value, as can be concluded from “landmark” trials such as the EORTC[19] and ESPAC-1[20].

Outline of the thesis

Since attempts to perform more radical surgery have failed to improve survival and cure rates (Chapter 2), several variants of adjuvant chemo- and radiotherapy have been explored. In the absence of an established adjuvant therapy, determination of the exact origin of a tumor was irrelevant when the first clinical trials were initiated. This is probably why the first adjuvant trials indifferently included both pancreatic ductal adenocarcinoma (PDAC) and non-pancreatic periamplary cancers (NPPC). With increasing understanding of the relevant subtypes of adenocarcinomas arising in the pancreatic head, later studies mainly included patients with PDAC. This brings us to the aim of this thesis; A correct classification of the different subtypes of cancer in the pancreatic head: those of pancreatic ductal origin (PDAC) and other non-pancreatic periampullary cancers (NPPC) and the implications for systemic treatment. This issue is discussed in Chapter 3.

The results of a randomised trial comparing adjuvant intra-arterial chemotherapy and radiotherapy to surgery alone are discussed in Chapter 4. Patients who underwent resection for NPPC or PDAC were stratified and randomised to either treatment. We focussed on long-term survival and pattern of recurrence, for both groups. In Chapter 5 we investigated factors that could predict survival after radical (R-0) resection for either NPPC or PDAC. All patients who underwent a true R-0 resection were analyzed. Furthermore, we performed a tissue microarray protein expression analysis for several growth factor receptors and oncogenes: HER-2, EGF-R, ER, PR, C-myc, p53, p16, RB-1, and chromogranin A as a neuroendocrine differentiation marker. In adition, further genetic analysis is discussed in Chapter 6 where we show the results of an ArrayCGH study comparing NPPC to PDAC. In order to investigate new treatment options for pancreatic cancer, in the final part of the thesis, Chapter 7 and 8, we further investigate the nature of pancreatic cancer in vitro. We describe the results of several in vitro experiments with type 1 interferons. We describe the effects and mechanisms of action. Not only on tumor cell but also on micro- and macrovascular endothelial cells.

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Chapter 2

Standard Resection of Pancreatic Cancer and the Chance for Cure

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Abstract

Introduction:
Pancreatic cancer is a devastating disease. Radical surgical resection offers the only chance for cure. In search of improved survival, more extended resections have been advocated. On the other hand, pyloruspreserving pancreaticoduodenectomy (PPPD) has emerged as an organ-preserving alternative to a standard Whipple resection (SW). However, its oncologic safety has been under debate. Furthermore, reports of increased rates of delayed gastric emptying have been made.

Methods:
We performed a Medline-PubMed search for relevant articles. In addition to nonrandomized reports, we found 3 randomized controlled trials (RCTs) comparing SW or PPPD with SW with extended lymphadenectomy and 4 RCTs comparing SW with PPPD.

Conclusions:
For resection of pancreatic cancer, SW and PPPD are probably equally effective. Extended resections are not warranted on the basis of the current evidence and should only be offered in an experimental setting.

Introduction

Since John Baptist Morgagni first described pancreatic cancer in the 18th century, it has been a challenge to treat this devastating disease. Radical surgical resection offers the only chance for cure. However, before the end of the 19th century, pancreatic surgery was rarely attempted. It was in 1898 that Allesandro Codivilla made a first attempt to perform a 1-stage pancreaticoduodenectomy. The pancreatic remnant was left intra-abdominally without enterostomy. The patient died 21 days postoperatively, with a "serous appearing fluid" leaking continuously from the wound. In 1903, Emil Theodor Kocher introduced his technique of duodenal mobilization, making pancreatic exposure easier. A few years later, in 1909, the first pancreaticoduodenectomy with preservation of the pancreatic remnant by performing a pancreaticoduodenostomy was performed by Walter Kausch. Whipple, Parsons, and Mullins further developed this technique now known as the "standard Whipple resection" (SW) and published their results in 1935. Since then, numerous changes were introduced in the technique of pancreaticoduodenectomy, many of them by Whipple himself. By using silk instead of catgut, the problems of dissolving sutures because of digestive enzymes were overcome. Furthermore, the introduction of vitamin K made it possible to perform a 1-stage procedure in jaundiced patients[1]. In the Western world, pancreatic cancer is still a lethal disease, and its incidence has increased over the years[2]. Unfortunately, only 10% of all patients are amenable to curative resection. Even after radical resection, survival rates remain low, and long-time survivors are sparse[3-5].

To date, adjuvant therapy has not substantially improved outcomes after surgery[6]. In 1973, Fortner[7] described a "regional pancreatectomy" in a search for more radical resection, hopefully leading to improved survival. The rationale for extended surgery can be found in the fact that locoregional failure is frequent after curative resection for pancreatic cancer (Table 1) [3,4,8-14]. Metastasis to retroperitoneal lymph nodes is common, and pancreatic cancer is known for its tendency to invade and grow along nerves surrounding the pancreas[15]. In the case of involvement of large adjacent vessels, a radical resection can be achieved by dissection and reconstruction. It has been shown that tumor-positive

resection margins are a negative prognostic factor for survival. This may be because of the radicality of the resection or biological behavior of the resected tumor itself[16].

As mentioned earlier, locoregional recurrence is observed frequently. However, most patients will succumb because of the development of distal metastasis (Table 1) and not local recurrence[13]. In fact, most patients will have micrometastases at the time of resection. In an overview by Vogel et al.[17] (Table 2), recent studies on this subject are reviewed[18]. By using advanced molecular detection techniques, it was revealed that tumor cells can be found pre- and perioperatively in peritoneal lavage fluids, the liver, blood, "tumor-negative" lymph nodes, and bone marrow of patients without "conventional" evidence of metastatic disease (Table 2).

Table 1:

Patterns of recurrence after curative resection

	Year	Method	N	Liver	Local	Both	Peritoneal	Lung	Bone	Other
Griffin et al [8]	1990	C	36	62	73		42			27
Westerdahl et al [9]	1993	A/C	74	92	86	78				
Kayahara et al [10]	1993	C	30	60	83		40			
Takahashi et al [11]	1995	C	25	80	100		56	56	24	
Sperti et al [12]	1997	C	78	62	72		6			
Klinkenbijl et al [4]	1999	C	135	50	53			9		27
Neoptolemos et al [14]	2004	C	158	34	35	43				
Hishinuma et al [13]	2006	A	27	50	75	46*	33	41		75

N = number of analyzed patients; C = clinical assessment of metastasis; A = assessment by autopsy;

A/C = autopsies and clinical assessment; R0-1 = macroscopically radical resection; Rx = margins undefined.

* Results after extended surgery, only 1 local recurrence without liver metastasis

Consequently, for most patients,pancreatic cancer is a systemic disease and resection merely a “lumpectomy.” Therefore, improved outcome after more extended resection remains uncertain. In other malignancies, such as breast cancer, a trend toward a less invasive, more restricted resection can be observed[19]. This is probably attributable to the emergence of effective systemic adjuvant therapy. Sadly, this is of limited value for patients with pancreatic cancer. Traverso and Longmire[20] along with Beger et al.[21] popularized the pylorus-preserving pancreaticoduodenectomy (PPPD), first performed by Watson in 1944[1]. The possible advantages of a PPPD over an SW (with partial gastrectomy) are less dumping, improved gastrointestinal functioning, and reduced jejunal ulceration[22]. Dumping is part of the postgastrectomy syndrome that consists of postprandial nausea, vomiting, dyspepsia, and diarrhea and represents frustrating and lasting symptoms for affected patients. PPPD is an overall simpler and shorter procedure[22]. On the other hand, delayed gastric emptying (DGE) has been reported and leads to episodes of nausea and vomiting as well as prolonged hospital stay. Furthermore, it increases the risk of aspiration and associated pneumonia. In the case of malignancy, oncologic efficacy of the resection can be argued in the case of a tumor-positive margin at the site of the pylorus or metastasis in gastric lymph nodes. This article discusses the value of extended surgery and lymph node dissection. The evidence to perform either an SW or PPPD is also evaluated.

Table 2: Presence of pre- and perioperative micrometastasis: a short resume of the review by Vogel et al.[17] PCR = polymerase chain reaction; IS immunostaining.

	Frequency (%)	Methods
Liver	77	PCR
Peritoneal	7-39	IS, PCR
Blood	4-100	IS, PCR
Bone marrow	8-66	IS, PCR
Lymph nodes	47-83	IS, PCR

Methods

We performed a Medline-PubMed search for relevant articles, and randomized controlled trials (RCTs) were selected for review. All other types of publications were included only if the authors found them to be relevant.

Results

Standard versus extended resection

We found 3 RCTs comparing SW or PPPD with SW with extended lymphadenectomy (Table 3)[23-26]. After report of a possible survival benefit for extended lymphadenectomy in patients with pancreatic and periampullary cancer[27], a first multicenter RCT was published by Pedrazzoli et al.[23]. There were 81 patients, all with pancreatic cancer, included in this RCT. The extended resection included resection of lymph nodes from the hepatic hilum and along the aorta from the diaphragmatic hiatus to the inferior mesenteric artery (IMA) and laterally to both renal hili. Furthermore, the celiac trunk and superior mesenteric artery were circumferentially cleared. No differences were found between groups in baseline characteristics. There were no differences in duration of the operation or blood loss, transfusion requirements, morbidity, mortality, or overall survival. In a postiori analysis, however, a significantly longer survival rate was found for node-positive patients. In a second RCT by the Johns Hopkins group[24], 299 patients with either pancreatic cancer or periampullary cancer were included. PPPD was compared with SW with extended lymphadenectomy. This extended operation included extended gastric resection and retroperitoneal resection from the hilum of the right kidney to the left lateral border of the aorta (horizontal axis) and from the portal vein to below the third part of the duodenum (vertical axis). Baseline characteristics were comparable between both groups. Eighty-six percent of the patients in the standard group underwent a PPPD. The operating time was significantly longer in the extended group. No differences were found with respect to blood loss, transfusion, or positive resection margins. Mortality was comparable. Morbidity was more frequent in the extended group, with higher rates of DGE, pancreatic fistula, and postoperative stay. There was no difference in overall survival.

A long-term follow-up of this study was also published[28]. Again, no survival benefit was observed. Another report of this trial covered the quality of life (QOL) analysis after extended surgery[25,28]. One hundred fifty questionnaires were sent to the 299 patients aforementioned in the Johns Hopkins trial[24]. A 70% return rate was achieved, possibly introducing considerable response bias. There was no difference in QOL after extended surgery compared with standard resection after 2 years. Farnell et al. included 132 patients in their trial[26]; only 79 patients proved to be resectable and were randomized for SW or extended surgery. All patients had pancreatic cancer, and all underwent a distal gastrectomy. Extended resection laterally included the hili of the kidneys, superiorly the celiac trunk, and distally the IMA (along the aorta). The hepatic artery, superior mesenteric artery, and celiac trunk were circumferentially cleared. The hepatoduodenal ligament was skeletonized up to the hilum of the liver. No differences were found between both groups in baseline characteristics, morbidity, and mortality. The operating time was longer for the extended resections. QOL analysis revealed a decrease 4 months postoperatively.

Standard Whipple versus PPPD

We found 4 RCTs comparing SW with PPPD (Table 4)[29-32]. Although survival analysis was reported in most studies, this was never a primary endpoint. The first RCT was published by Lin and Lin[31]. From August 1994 to 1997, 31 patients with pancreatic and periampullary cancer were randomized to undergo either an SW or PPPD (15 vs 16). There were no differences in baseline characteristics, operating time, blood loss, mortality, or morbidity. Although more cases of DGE were reported in the PPPD group, this was not statistically significant (p=.08). In a second report by the same author[32], 36 patients were recruited in a period from July 1994 to 2002. Thirtythree patients were randomized (14 PPPD and 19 SW, all pancreatic cancer). It is unclear if this study is a different trial by itself or a follow-up and/or a subanalysis of the previous study. However, no differences between groups were reported in baseline characteristics, operating time, blood loss, mortality, morbidity, and overall survival. Significantly more cases of DGE were reported in the PPPD group (p=.003). In the largest reported study comparing SW with PPPD, 170 consecutive patients were included[30]. Most patients had either pancreatic or periampullary cancer; 29 patients with benign

and 7 patients with neuroendocrine lesions were excluded from survival analysis. Baseline characteristics were comparable, and no differences in operating time, blood loss, mortality, morbidity, and DGE were found between the 2 groups. There was no survival benefit for either group (p=.90). Another large RCT was reported by Seiler et al.[29]. In this trial, 130 patients (pancreatic and periampullary cancer, 20 patients with benign lesions were excluded from survival analysis) were analyzed. Baseline characteristics were comparable. The operating time was longer in the SW group, as was blood loss. No differences were observed between the groups regarding DGE, mortality, morbidity, and survival. A QOL assessment did not reveal significant differences.

Table 3: Standard Whipple resection (SW) or pylorus-preserving pancreaticoduodenectomy (PPPD) versus SW with extended lymphadenectomy

	Year	N		QOL	Survival	Hospital stay	Operating time	Blood loss	Mortality	Morbidity
Pedrazzoli et al [23]	1998	81	PC	-	=	=	=	=	=	=
Yeo et al [24]	2002	299	PC+PAC	*	=	> (p=0.003)	> (p=0.002)	=	=	> (p=0.01)
Nguyen et al [25]	2003	150	PC+PAC	=	=	> (p=0.01)	> (p=0.001)	=	=	=
Farnell et al [26]	2005	79	PC	< (p<0.05)	=	=	> (p=0.01)	-	=	=

PAC = periampullary cancer; PC = pancreatic cancer; S = survival; = no difference; - = not reported; < = favors SW with extended lymphadenectomy; > =favors SW/PPPD.* Quality of life is reported separately.

Table 4: Pylorus-preserving pancreaticoduodenectomy (PPPD) versus standard Whipple resection (SW)

	Year	N		QOL	DFS	Survival	DGE	Hospital stay	Operating time	Blood loss	Mortality	Morbidity
Lin and Lin [31]	1999	31	PC+PAC	-	-	-	> (p=0.08)	-	=	=	=	=
Tran et al [30]	2004	170	PC+PAC	-	=	=	=	=	=	=	=	=
Seiler et al [29]	2005	130	PC+PAC	=	-	=	=	=	< (p=0.001)	< (p=0.041)	=	=
Lin et al [32]	2005	30	PC	-	-	=	> (p=0.003)	=	=	=	=	=

S = survival; DFS = disease-free survival; DGE = delayed gastric emptying; PAC = periampullary cancer; PC = pancreatic cancer; = =no difference; - = not reported; > = favors SW; < = favors PPPD.

Discussion

Radical resection offers the only chance for cure in patients with pancreatic head and periampullary cancer. Adjuvant therapy remains of marginal benefit[6]. The technique of pancreaticoduodenectomy has evolved considerably since Codevilla, Kausch, and Whipple. Currently, a resection with curative intent can be attempted with in-hospital mortality of less than 5%[33]. This is a major step forward compared to results only 2 decades ago when mortality rates of 25% or more were reported. There continues to be a difference in mortality between low- and high-volume centers[34,35]. Centralization of pancreatic surgery to highvolume centers has been widely recommended; however, implementation of tertiary patient referral remains difficult[35].

Codivilla's patient probably died of pancreatic secretions leaking into the abdominal cavity and fistulating to the wound. Because no replacement therapy for insulin and digestive enzymes was available, the conservation of a pancreatic remnant was unavoidable. Even at the present time, the pancreatic enterostomy remains the Achilles' heel of a pancreaticoduodenectomy. A major part of the morbidity and mortality can be attributed to pancreatic anastomotic failure. Major improvements have been made in the treatment of diabetes mellitus and digestive enzyme replacement in the last decades. Nonetheless, conservation of a pancreatic remnant is considered to be essential in light of the "curative" intent of a pancreaticoduodenectomy.

Several attempts have been undertaken to technically improve the reliability of the anastomosis. The optimal anastomotic site, gastric or jejunal, remains unclear[36]. The only RCT comparing these 2 anastomotic sites showed no difference in outcomes between the 2 techniques[37]. Occlusion of the pancreatic duct as an alternative for pancreaticoenterostomy does not reduce abdominal complications and in the long run increases the risk of developing diabetes[38]. Perioperative treatment with somatostatin and its analogues has proved to be successful in reducing the incidence of fistula[39]. In pursuit of superior surgical results in terms of survival, more extended resections have been advocated.

Extended resection combines an SW (including partial gastrectomy) with resection of retroperitoneal soft and lymphatic tissue ranging from diaphragmatic hiatus to

the IMA and laterally to the hili of the kidneys. In several retrospective studies, a survival benefit was reported. In a retrospective study by Ishikawa et al.[27] published in 1987, 59 patients were analyzed. An improved survival rate from 13% to 38% in favor of the extended resections was noted. It was concluded that small tumors confined to the pancreas may benefit most from extended resection. A major shortcoming of this study, besides being nonrandomized, was that the 2 groups were recruited in different time periods. Furthermore, comparison of both groups was complicated by inequalities in pathological staging. In opposition, another retrospective analysis by Satake et al.[40] in 1992 of 185 patients resected for tumors smaller than 2 cm showed no difference in survival. Another retrospective report[41] of 501 patients also did not find a survival benefit for extended surgery. In a prospective but nonrandomized report by Henne-Bruns et al.[42], 72 patients were analyzed; 26 underwent standard resection and 46 an extended retroperitoneal lymphadenectomy. No benefit was found for extended surgery. Portal vein involvement was a predictor of poor outcome, even after radical resection and reconstruction. We found reports of 3 RCTs on extended versus standard resection (Table 3). The study by Pedrazzoli et al.[23] showed no benefit for extended resection. However, in a postiori analysis, a survival benefit was found for node-positive patients. The study by Farnell et al.[26] showed decreased QOL 4 months postoperatively and increased operative time. The largest trial from the John Hopkins group was reported in several publications. Primary results, QOL, and long-term follow-up were all published separately. Although a trend was observed toward improved survival in an underpowered subgroup analysis, the overall conclusion showed no benefit for extended resection. Disadvantages of extended surgery were longer operating time, increased morbidity, and increased hospital stay. All reports cover “extended” resections. However, the extent of this resection varies between the respective studies. Farnell et al.[26] skeletonized the hepatoduodenal ligament up to the hilum of the liver, whereas the others did not. Furthermore, resection or clearance of major adjacent vessels is rather variable. Although the extent of surgery is not standardized between these studies and therefore direct comparison is impossible, these studies offer the only level 1 evidence for the extent of resections. It can be argued that these studies are underpowered to detect a (small) benefit of extended surgery. Recently, Pawlik et al.[43] published their power analysis. They concluded that an RCT should include more than 200,000 patients in each arm

to detect their expected difference. It is clear that such a study is neither feasible nor useful. All present RCTs confirm that there is no expected survival benefit for extended surgery. Nevertheless, there is an increase in costs of hospital stay and operation time. Furthermore, treatment-related morbidity may be higher and QOL lower. On the basis of this evidence, extended resections should not be performed.

Since the introduction of PPPD, debate has continued on its value and oncologic safety. Possible advantages of PPPD are improved long-term gastrointestinal functioning as well as simplification of the operation, possibly leading to shorter operations and less blood loss. DGE has been reported to be associated with PPPD in several retrospective reports. The first randomized study by Lin and Lin[31] showed no differences between SW and PPPD. Their second article from 2005 showed data from a different group of patients recruited in the same period[32]. Significantly more patients were found to have to have DGE in the PPPD group. Both large RCTs by Seiler et al. and Tran et al.[29,30] showed that both procedures have similar results in terms of morbidity, mortality, and survival. The aforementioned trials represent strong evidence that SW and PPPD are equally effective in the treatment of pancreatic and periampullary cancer from an oncologic point of view. However, one must be cautious to draw definite conclusions because survival was not their primary end point. PPPD has no clear advantages other than being a simpler procedure. DGE remains a challenging problem for either technique. Recently, an RCT and a prospective analysis were published showing that DGE can be reduced dramatically by making an antecolic duodenojejunostomy during PPPD.[44,45]

Pancreatic cancer, in general, is not curable by local therapy (surgery) only. A “curative” resection should therefore be associated with minimal mortality and morbidity. More than ever, QOL should be a primary end point in evaluation of surgical treatment.[46,47] Ex ended surgery is not warranted on the basis of the currently presented evidence and should only be offered in an experimental setting. An “evidence-based” standard resection for cancer of the head of the pancreas should comprise a PPPD without extended resection of soft tissue and lymph nodes. Probably, an antecolic duodenojejunostomy is superior in terms of DGE. For most, if not all patients, pancreatic cancer represents a

systemic disease requiring systemic therapy. It is clear that our future efforts should be focused on finding more effective systemic (neo-) adjuvant therapies.

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Chapter 3

Histological and Molecular Subclassification of Pancreatic and Non-Pancreatic Periampullary Cancers; Implications for (Neo) Adjuvant Systemic Treatment.

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Abstract

The benefit of adjuvant chemotherapy for resected pancreatic ductal adenocarcinoma (PDAC) has been confirmed in randomized controlled trials. For non-pancreatic periampullary cancers (NPPC), originating from the distal bile duct, duodenum, ampulla and the papilla of Vater, the role of adjuvant therapy remains largely unclear. In this review we describe methods to distinguish PDAC from NPPC by means of readily available and recently developed molecular diagnostic modalities. We also discuss the difficulties of reliable pathological determination of the exact origin of these cancers. We consider the possibility of the unintended inclusion of NPPC in the most important adjuvant trials on PDAC and subsequent implications for interpretation of the results. We conclude that correct determination of the origin of periampullary cancers is essential for clinical management and should therefore be systematically incorporated in clinical practice and future studies.

Introduction

Despite different origins, all adenocarcinomas in the periampullary region have a similar clinical presentation, mostly by obstructive jaundice. The majority of these tumors are of pancreatic ductal origin, less frequently tumors in the periampullary region may arise from the distal common bile duct, ampulla of Vater or duodenum. Collectively, and sometimes confusingly, these are known as periampullary cancers. Perhaps it would be better to describe these tumors as non-pancreatic periampullary cancers (NPPC), since pancreatic ductal adenocarcinoma (PDAC) can also occur around the ampulla. Although histologically very similar, NPPC carry a more favorable prognosis. The classical hypothesis is that NPPC are usually diagnosed at an earlier stage because of their anatomical location, leading to early jaundice and clinical manifestation. For this reason NPPC may more frequently be amenable to radical surgical resection than their pancreatic counterparts. On the other hand, NPPC may represent a separate family of tumors with a different biological behavior. This hypothesis is supported by the fact that, after correction for tumor size, positive lymph nodes and stage, the survival for these NPPC remains superior[1,2].

Currently, radical surgery offers the only chance for long-term survival and cure. All adenocarcinomas in the pancreatic head or periampullary region require a similar pancreatoduodenectomy, in an attempt to radically remove the tumor and regional lymph nodes[3]. Even after radical resection, survival remains poor, particularly for PDAC. Median survival after radical resection for these tumors is about 15 months for PDAC and 45 months for NPPC [4-7].

To improve survival after resection for both PDAC and NPPC, several variants of adjuvant chemo- and radiotherapy have been explored. In the absense of an established adjuvant therapy, determination of the exact origin of a tumor was irrelevant when the first clinical trials were initiated. This is probably why the first adjuvant trials indifferently included both PDAC and NPPC. With increasing understanding of the relevant subtypes of adenocarcinomas arising in the understanding of the relevant subtypes of adenocarcinomas arising in the pancreatic head, later studies mainly included patients with PDAC. As

a consequence trials on NPPC and adjuvant therapy are sparse. Only two randomized trials included NPPC in the last decade[8,9].

The objective of this article is to review histopathological and molecular modalities to clearly distinguish PDAC and NPPC. Hereafter we review the current evidence for adjuvant therapy in relation to the difficulties of reliable pathological determination of the exact origin of these cancers.

Defining pancreatic (PDAC) and non-pancreatic cancers (NPPC) of the periampullary region.

Several definitions of periampullary cancer co-exist[10]. Most authors include cancers originating from the distal common bile duct, ampulla and papilla of Vater or duodenum near the papilla. However, some include all cancers located within 2 cm of the papilla of Vater, this definition also includes "periampullary" cancers of pancreatic ductal origin. The term "periampullary cancer" should only be used in the pre-operative work-up to describe a tumor in the periampullary region on imaging. In pathology reports, the terms "periampullary" or "pancreatic head" should be avoided since these only describe gross location of the tumor but not origin. Furthermore, "periampullary" pancreatic cancers should correctly be classified as PDAC[11].

Pathological assessment often results in misclassification of periampullary tumors

Correctly classifying the origin of "periampullary" tumors can be difficult, especially for large tumors with distorted anatomy. Pathologists were recently surveyed on their approach to determine tumor origin in pancreatoduodenectomy specimens: 20% reported that they routinely determine the origin of periampullary tumors, 67% tries to distinguish true PDAC from NPPC, and 13% did not[12]. This is not surprising, in the absence of effective (neo)adjuvant chemotherapy, pathologist were previously less inclined to meticulously subclassify periampullary cancers. Tumors were classified as periampullary cancer, or misclassified as NPPC or PDAC. This inconsistency in pathology reporting may be the cause of

the differences in pathological and survival data, clearly illustrated by Verbeke et al. in their recent publication, describing the wide range in relative incidence of pancreatic, ampullary and distal bile duct cancer in pancreatoduodenectomy series[13]. Another study, by Pomianowska et al[14], revealed that NPPC are commonly misclassified as PDAC during pathological assessment. In this study two experienced pathologists re-reviewed a series of 207 histopathology reports. In 27% of cases the diagnosis was changed, mainly from PDAC to distal bile duct cancer.

In a Finnish cancer registry study, 4922 patients with PDAC were analyzed. Only 89 patients survived more than five years. Of these 89 patients 45 actually were incorrectly registered and had not been diagnosed with PDAC in the first place. Another 18 patients did not have any histological confirmation. In the 26 patients recorded as having histologically proven PDAC, re-evaluation of histological specimens confirmed PDAC in only 10 patients and 8 patients turned out to have distal bile duct or ampullary cancer[15].

How can these cancers be distinguished?

In order to try to distinguish PDAC from NPPC, several diagnostic modalities are available: histology, immunohistochemistry, protein and growth factor (receptor) expression, genetics and micro-RNAs. Most of these methods are widely available, can be used with paraffin embedded tissue, and are commonly used for other indications in most pathology laboratories.

Histology and immunohistochemistry

Standard microscopic histological assessment by itself cannot differentiate between NPPC or PDAC. Two histological subtypes, intestinal and pancreatobiliary differentiation can be identified in both PDAC and NPPC by microscopic assessment of the epithelium morphology[16]. In addition, several immunohistochemical markers such as CK20, CDX2 and MUC1 are available. CK20 is a marker of intestinal-epithelial origin[17]. CDX2 is a transcription factor associated with intestinal differentiation and is almost exclusively expressed in

intestinal epithelium. MUC1 is a glycoprotein, mainly expressed in tumors of the pancreatobiliary type[18]. Pancreatobiliary differentiation may indicate more aggressive biological behavior. When NPPCs with pancreatobiliary differentiation are compared to PDACs, survival is similar, whereas intestinal differentiation in PDAC is associated with a longer survival[16]. Although pancreatobiliary and intestinal differentiation may divide NPPCs and PDACs in two distinct and probably relevant groups, it cannot be used to differentiate between PDAC and NPPC since both NPPC and PDAC can have an intestinal or pancreatobiliary differentiation.

Proteins, Growth factors and Receptors

The epidermal growth factor receptor (EGFR) , also known as c-erbB1, is part of the erbB-family and its expression is often upregulated in various malignancies[19]. Over expression is common in PDAC but not in NPPC[2,20]. Furthermore, the expression of the EGFR may be associated with poor survival[2]. Also two other receptors of the erbB-family, namely c-erbB2 and c-erbB3, are known to be expressed in a higher level in PDAC compared to ampullary carcinoma[20,21]. Mucins (MUC) are high molecular weight, O-glycosylated proteins[22,23]. The MUC-family includes three subgroups: gel-forming, soluble and transmembrane mucins[23]. The transmembrane MUC1 and MUC4 are known to interact with respectively erbB1 and erbB2. MUC1 is reported to be overexpressed in PDAC[24] and MUC4 is expressed in pancreatic cancer, but not in normal pancreatic tissue[25-27]. Also an upregulation of the transmembrane MUC3 and the gel-forming MUC2 is associated with pancreatic cancer[26,28]. The gel-forming mucin MUC5ac is a marker for biliary tract cancer[29]. MUC3, 4 and 5ac are overexpressed in ampullary malignancies and MUC3 is an independent predictor of poor survival[30]. The expression of mucins differs between tumors with pancreaticobiliary or intestinal differentiation[31,32]. MUC1 and MUC4 are abundantly expressed in tumors with a pancreaticobiliary differentiation whereas MUC2 indicates intestinal differentiation[31]. The combination of positive expression of MUC1 and a negative expression of CK20 is distinctive for tumors with a pancreaticobiliary differentiation and is prognostic for dismal survival[32]. An expression profile of MUC1-/CK20+ is typical for intestinal differentiated tumors and is associated with a better prognosis[32].

Pancreatic cancers are characterized by a strong desmoplastic reaction and hypoxia due to poor vascularisation. Consequently, angiogenesis quantified by micro vessel density (CD31 staining) seems more prominent in NPPC compared to PDAC[33]. Another protein, the transcription factor "high mobility group A1", is expressed at higher levels in PDAC than NPPC. Interestingly it is associated with poor survival when present in NPPC[34].

Genetics

Known genetic alterations in the development of PDAC include activating mutations of the K-RAS oncogene and loss of the tumor suppressor genes SMAD4 and P53[35]. Mutations of K-RAS are more frequently present in PDAC (up to 90%) than in ampullary cancer (13% to 50%). Besides, K-RAS mutations at codon 12 is typical for PDAC whereas ampullary tumors typically harbor codon 13 mutations[36]. Direct genetic comparison by means of comparative genomic hybridization was studied in a small group of resected pancreatic and ampullary cancers and revealed several specific differences in chromosomal changes[37]. In a study published by Overman et al. [32] fresh-frozen resected tumors were studied by means of a full genome array[38]. They were able to discriminate PDAC from NPPC based on specific profiles. These results are promising, but the study groups remain small and the results require external validation. However, it is foreseeable that in the future it might be possible to define the origin of these tumors based on genetic profiling.

Micro-RNA

Micro-RNAs are short, non-coding, strands of RNA. Micro-RNAs are thought to be implicated in the epigenetic regulation of (cancer) cells. Many relevant micro-RNAs have been identified in PDAC and NPPC. The exact role of these micro-RNAs remains uncertain. The analysis of micro-RNA is still experimental, expensive and not widely available. Several reports suggest that micro-RNA analysis may be able to discriminate certain subtypes of NPPC and PDAC. Further validation is needed to confirm these results[39,40].

Randomized controlled trials on adjuvant chemo- and radiotherapy

The currently available evidence concerning the value of adjuvant chemotherapy for resectable PDAC and NPPC is based on a few large studies. Although these studies were performed with great effort and were carefully designed, most studies suffer from methodological flaws and incomplete pathology reporting. The most important trials are listed in Table 1.

The first report on adjuvant chemoradiotherapy for PDAC was the GITSG study randomizing between adjuvant 5-fluorouracil-based chemoradiation and observation[41]. A survival benefit was observed for the intervention group. The pathological assessment of the specimen was reviewed by two independent pathologists. NPPCs were explicitly excluded. For many years this study has been the only randomized trial in support of adjuvant chemoradiotherapy in PDAC. In 1993 Bakkevold et al. presented data of a trial comparing adjuvant doxorubicin, mitomycin and 5-fluorouracil to surgery alone[42]. There was no formalized pathology control. Remarkably, only patients with bile duct cancer were excluded from this study. A short-term improvement in survival was found in the intervention group. Nevertheless, 5-year survival was similar between groups.

In the European Organization for Research and Treatment of Cancer (EORTC) Trial 40891[5,43], patients with either PDAC or NPPC were randomized between 5-fluorouracil-based chemoradiotherapy and observation. Central pathology review was performed by one dedicated pathologist. Periampullary cancer was defined as distal bile duct, papilla of Vater and duodenal cancer. No survival benefit of adjuvant therapy was observed for either PDAC or NPPC.

The first European Study Group for Pancreatic Cancer (ESPAC) trial was designed with a two-by-two factorial design[44]. Patients with PDAC were randomized to either adjuvant chemotherapy (5-FU with folinic acid), chemoradiotherapy or observation. There was no central pathology review. A “potential” survival benefit was observed for chemotherapy, not for chemoradiotherapy. However, there was no central control of the radiation quality in the 61 participating centers. Furthermore, the lack of central pathology review

has been widely criticized for compromising accurate reporting on resection margins. The first German Charité Onkologie (CONKO) trial demonstrated a disease-free survival benefit for gemcitabine-based adjuvant chemotherapy as compared to observation alone for PDAC. Pathological examinations were performed locally, without central review. Slow accrual in the first months led to broadened inclusion criteria and randomization of both radically (R0) and microscopically irradically (R1) resected tumors. The first analysis did not show improved survival, yet long-term results showed significantly improved median overall survival[45,46]. The Japanese Study Group of Adjuvant Therapy for Pancreatic Cancer (JSAP) [47] combined 5-fluorouracil with cisplatinum verus surgery alone for PDAC. Patients with a positive resection margin were excluded. Three pathologists reviewed the first 18 cases together, the other 71 were reviewed by a local pathologist. Morak et al.[8] compared adjuvant intra-arterial chemo radiotherapy to surgery alone. Periampullary cancers, defined as distal common bile duct and ampullary tumors were included and prestratified. One pathologist reviewed all specimens centrally. No therapeutic benefit of chemoradiotherapy for PDAC was seen. However, during follow-up fewer liver metastases were observed in the NPPC group. In the Radiation Therapy Oncology Group (RTOG) trial[48], adjuvant gemcitabine was compared to 5-fluorouracil both with concurrent radiotherapy. Eligibility criteria were adenocarcinoma of the pancreas and gross total tumor resection. There was no central pathology review. All patients were restaged after surgery. Surgical margins were categorized as negative, microscopically positive or unknown. Of all pathology reports 25% contained no information regarding radicality of the resection. There was no statistically significant difference in survival. Neoptolemos et al. reported the results of the ESPAC-3 pancreatic cancer[49] and periampullary cancer[9] trials. In the pancreatic cancer trial adjuvant 5-fluorouracil was compared to gemcitabine in 1088 patients. Patients were eligible after macroscopic radical resection for PDAC. All specimens were analyzed by local pathologists in 159 centers across the world. There was no central pathology review. Survival did not differ between groups.

In the periampullary cancer study all pathology synoptic reports were reviewed by two experienced pathologists, but these pathologists did not review the actual slides and macroscopy[9]. In this study, the authors were unable to demonstrate a survival benefit of adjuvant chemotherapy for NPPC. It should be recognised that patients were not prestratified according to NPPC subtypes. In multivariate analysis, adjusting for these NPPC subtypes, a modest benefit was found for adjuvant gemcitabine based therapy.

Based on the results of the aforementioned trials, consensus has been reached that adjuvant chemotherapy for PDAC prolongs disease-free and overall survival. The role of (chemo) radiotherapy remains controversial. For NPPC there is no consensus regarding adjuvant therapy. NPPC were included intentionally in only four adjuvant trials (Table 1) and gemcitabine or 5-FU appears to be of little or no benefit for resected NPPC. In the randomized, but palliative, ABC-2 trial on advanced bile duct cancer (intra- and extrahepatic, locally advanced or metastatic cancer) and the aforementioned trial by Morak et al. cisplatinum was added to the regimen (Table 1) [8,50]. This may prove to be effective in the treatment of bile duct cancers. Furthermore, based on the subgroup analysis of NPPC in the Morak study, regional infusion of cisplatinum based chemotherapy may be even more effective and prevents occurrence of liver metastases and may lead to subsequent improved survival.

Table 1, Summary of most important randomised trials on adjuvant therapy for NPPC and PDAC.

	Ref.			N	Multi-center	Centers	incl. period	NCY	Central pathology review	R-0 %*	Definition NPPC	Inclusion of NPPC	Restaging	Study regimen	Median Survival (months)	p-value
Neoptolemos *et al* 2010	49	ESPAC-3	CT	1088	Yes	159	7	1.0	No	65%	No	No	No	5FU vs GEM	23 vs 23	0.39
Neoptolemos *et al* 2012	9	ESPAC-3 (NPPC)	CT	428	Yes	100	8	0.6	No	84%	Yes	Yes	No	5FU vs GEM vs SO	39 vs 46 vs 35	0.23
Ueno *et al.* 2009	51	JSAP-2	CT	118	Yes	10	3	1.7	No	84%	No	No	No	GEM vs SO	22 vs 18	0.19
Kosuge et al. 2006	47	JSAP	CT	89	Yes	11	8	1.2	No	100%	No	No	#	5FU+CIS vs SO	13 vs 16	0.94
Oettle *et al.* 2007	45/46	Conko-001	CT	368	Yes	88	6	0.6	No	83%	No	No	No	GEM vs SO	23 vs 20	0.01
Bakkevold et al. 1993	42		CT	61	Yes	20	3	0.4	No	100%	Yes	Yes	No	AMF vs SO	23 vs 11	0.10
Regine *et al.* 2008	48	RTOG	CRT	451	Yes	164	4	0.4	No	42%**	No	No	Yes	GEM or 5FU + 5FU&RT	21 vs 17	0.09
Morak *et al.* 2008	8		CRT	120	No	1	7	17.1	Yes	84%	Yes	Yes	Yes	CAI/RT (5FU-CIS-Mx) vs SO	19 vs 18	0.25
Neoptolemos *et al.* 2001	44	ESPAC-1	CT/ CRT	541	Yes	61	6	1.3	No	82%	No	No	No	5FU vs SO(2x2): 5FU+RT vs SO(2x2):	17 vs 16 16 vs 18	0.19 0.09
Klinkenbijl *et al.* 1999	5/43	EORTC	CRT	218	Yes	29	8	1.1	Yes	80%	Yes	Yes	No	5FU+RT vs SO	19 vs 25	0.21
Kalser et al. 1985	41	GITSG	CRT	43	Yes	#	8	#	Yes	100%	Yes	No	No	5FU+RT vs SO	20 vs 11	0.04

Symbols: * averaged over groups,** 25% unknown margins,

unclear from methods, NCY: Number/Center/year, Restaging: patients restaged after surgery prior to adjuvant therapy.

SO: surgery only, 5FU: 5-flourouracil, CIS: Cisplatin, GEM: Gemcitabine, AMF: 5FU, doxorubicin, mitomycin, CAI: celiac axis infusion, Mx: Mitoxantrone, 2x2: two-by-two factorial design

Conclusions and future considerations

Most of the studies summarized in Table 1 have been extensively discussed in previous reviews. All these studies have their specific limitations: randomization of patients after surgery, longstanding accrual of patients, a small number of patients included at each individual center or unconventional designs including three study arms and two-by-two factorial designs.

Besides these common limitations, there is another important issue concerning trials on PDAC and adjuvant therapy: difficulties in correct determination of the origin of a pancreatic head mass. It is clear that without standardized and centralized pathology assessment it is very likely that the frequently underdiagnosed NPPCs were unintentionally included in the analysis of adjuvant therapy for PDAC.

Consider the possibility of underestimation of bile duct cancers in these trials. What would be the effect on survival and what would be the effect of adjuvant therapy if these and other NPPCs were indeed accidentally included?

A similar observation can be made concerning studies on neo-adjuvant or palliative therapy for PDAC. It is impossible to rule out the inclusion of NPPCs in these studies, since patients are included without complete pathological assessment of the primary tumor. This should be taken into account before extrapolating these results to the adjuvant setting and vice versa. Furthermore, with current developments of endo-ultrasound guided biopsy methods, actual tissue cores can be sampled. These could be used for analysis of known markers and new techniques in development such as the micro-RNAs. When these techniques become more refined, perhaps they could give some direction to what would be an optimal (neo)adjuvant or palliative regimen.

Currently, there is no validated molecular or biological classification for NPPC or PDAC and therefore we are forced to classify first by means of macroscopic and microscopic histological assessment. However, a few simple and inexpensive improvements could be easily incorporated: Standardized and protocolized pathological examination, for example by simple axial slicing, may give very reproducible results. In addition, with current technology, high resolution macroscopy

(i.e. from slices) and microscopy images can easily be obtained and shared for central review. Additionally, simple and widely available staining of intestinal markers such as CK20 and CDX2 should be added to identify and possibly stratify for the currently most relevant histo-molecular subgroups: intestinal and pancreatobiliary differentiated cancers. Future studies probably should also incorporate analysis of other molecular or genetic markers (or at least collect and store tissue samples), although a clear direction, based on the current literature, is difficult to give. The key point is that the origin of a “periampullary” cancer is an essential part of the multidisciplinary discussion whether a patient may benefit from adjuvant therapy in terms of disease free and overall survival. We conclude that determination of the origin of a periampullary cancer (NPPC subtypes and PDAC) is essential for optimal clinical management and should therefore be systematically incorporated in clinical practice and future studies.

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Supplement to Chapter 3

Resection Margin Involvement and Tumour Origin in Pancreatic Head Cancer

Joris I. Erdmann and Casper H.J. van Eijck

The British Journal of Surgery 2013;100(2):299.

A letter to the Editor concerning:

Verbeke CS, Gladhaug IP. Resection Margin Involvement and Tumour Origin in Pancreatic Head Cancer. The British journal of surgery, 2012;99(8):1036-1049.

Letter to the Editor

Sir,
We were interested to read the recent publication in the BJS by Verbeke and Gladhaug[1] .

The current guidelines on adjuvant chemo- and radiotherapy after resection of pancreatic and periampullary cancers are based on the results of only a few large randomised trials[2].

Interestingly, periampullary cancers were only intentionally included in two trials[3,4]. There is no mention or definition of periampullary cancer, or for that matter pancreatic head cancer located nearby the ampulla in other trials. The correct classification of periampullary tumours may be compromised by the lack of precise pathological and clinical definition in almost all multi-centre studies. After reading this paper, one can imagine the difficulties encountered in determination and consequent exclusion of non-pancreatic “periampullary” tumours, especially when considering larger tumours. As a result it is inevitable that periampullary tumours have been included as pancreatic cancers and vice versa. It would be better to avoid the terms “periampullary” or “pancreatic head” as these only describe the gross location of the tumour and not the exact origin.

Although it would be prudent to classify some tumours as of “uncertain” or “unknown” origin, in clinical practice most will probably be classified as “pancreatic head tumour”. An understandable pragmatic solution since radical resection used to be the only proven treatment modality, and subtyping was less relevant. However, in the current era of neo-adjuvant therapy and even for palliative therapy, determination of the origin of a pancreatic head tumour is of increasing importance.

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Author reply:

Sir,
We agree that in pancreatoduodenectomy specimens correct identification of the cancer origin, ie. pancreatic, ampullary or distal common bile duct, is of key importance for clinical trials. We also share their opinion that this aspect has not been given due attention and that the likely inaccuracy of tumour origin identification in the past has been ignored.

Central pathology review is essential for quality assessment and should be an integral part of all multicentre clinical trials. As identification of the cancer origin is mainly based on the exact tumour location, the traditional microscopic slide review is to be complemented with review of the macroscopic findings, i.e. photographs of the relevant specimen slices. Pathology protocols should therefore provide detailed information on the gross appearanceof specimens and the macroscopic photo documentation. Difficulties in correct assignment of cancer origin have been demonstrated in a recent study[1].

A category of cancers of 'uncertain' origin risks being over-used and reduce the required diagnostic commitment. Only in a small proportion of pancreatic head cancers is the origin difficult to identify, and a 'most likely' diagnosis following judicious macro- and microscopic assessment represents probably more robust data than a less well-reflected assignment to the 'uncertain' category.

Markers specific for the various cancer origins have not been identified. In recent years the picture has become more complex with the observation that adenocarcinoma of pancreatobiliary and intestinal morphology can develop from each of the sites of origin[2].

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Chapter 4

Long-term Survival after Resection for Non-Pancreatic Periampullary Cancer Followed by Adjuvant Intra-arterial Chemotherapy and Concomitant Radiotherapy

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Abstract

Background: There is no consensus regarding the optimal adjuvant treatment after resection of non-pancreatic periampullary adenocarcinoma (NPPC; distal common bile duct, ampulla, duodenum).

Objectives: The present study was conducted to evaluate the impacts on longterm survival and recurrence of celiac axis infused adjuvant chemotherapy (CAI) and concomitant radiotherapy (RT) in patients submitted to resection for NPPC or pancreatic ductal adenocarcinoma (PDAC) in a randomized controlled trial.

Methods: A total of 120 patients with PDAC (n = 62) or NPPC (n = 58) were prestratified at a ratio of 1:1 for tumour origin and randomized. Half of these patients were treated with adjuvant CAI/RT and the other half were treated with surgery alone. Follow-up was completed for all patients up to 5 years afterresection or until death.

Results: There was no survival benefit in either the whole group (primary endpoint) or the PDAC group after CAI/RT. In the NPPC group, longterm survival was observed in 10 patients in the CAI/RT group and five patients in the control group: median survival was 37 months and 28 months, respectively. The occurrence of liver metastases was reduced by CAI/RT from 57% to 29% (p = 0.038). Cox regression analysis revealed a substantial effect of CAI/RT on survival (hazard ratio: 0.44, 95% confidence interval 0.23–0.83; p = 0.011).

Conclusions: This longterm analysis shows that median and longterm survival were improved after CAI/RT in patients with NPPC, probably because of the effective and sustained reduction of liver metastases. The present results illustrate that NPPC requires an adjuvant approach distinct from that in pancreatic cancer and indicate that further investigation of this issue is warranted.

Introduction

The treatment of adenocarcinoma of the pancreas and periampullary region remains a challenge. Even for those few patients who are amenable to resection with curative intent overall survival remains poor. Most tumors arise in the pancreatic head near the ampulla of Vater. The majority of these tumors are of pancreatic ductal origin (PDAC). Less often tumors in the pancreatic head region arise from the distal common bile duct, ampulla of Vater and duodenum, collectively known as periampullary cancers[1]. Although histologically very similar, these tumors bear a more favorable prognosis. The common assumption is that these tumors are diagnosed at an earlier stage because they lead to jaundice early due to their anatomical location. For these reasons non-pancreatic periampullary tumors (NPPC) may more frequently be amenable to surgical resection than their pancreatic counterparts. On the other hand evidence is increasing that NPPC are a separate family of tumors with a different biological behavior.

Currently, evidence supports adjuvant chemotherapy after resection for PDAC[2]. There is no clear evidence to recommend adjuvant therapy after resection for NPPC[3,4]. We conducted a single center randomized trial in which we compared adjuvant treatment with intra-arterial chemotherapy and concomitant radiotherapy (CAI/RT) to surgery alone. The choice for CAI was supported by several small phase 1-2 trials, showing promising results for a 5-FU, mitoxantrone and cisplatinum based CAI regimen in advanced and resected pancreatic cancer[5-7]. The rationale to add radiotherapy was to prevent local recurrence.

Similar to the well-known EORTC trial[3,8] and - at the start of the trial - without any available evidence-based standard adjuvant treatment for either group, patients with NPPC and PDAC were equally included in this trial. Patients were pre-stratified for either aforementioned cancer after surgical resection. In 2008, shortly after inclusion of the last patient, we published the first results[9]. We could not demonstrate a survival benefit for PDAC. However, adjuvant CAI/RT reduced the number of patients with NPPC who developed liver metastases. A discussion followed after our publication whether this was just a temporary effect or in fact

could lead to a actual better long-term survival. Here we present the long-term data of this randomized controlled trial in which all patients have been followed for at least five years or up to death, with special focus on NPPC.

Methods

The study design was described in the primary analysis in detail[9]. The trial was approved by the local medical ethics committee. Patients were randomized after resection into two groups: CAI/RT or surgery alone. All specimens were reviewed by one specialized pathologist (HvD), graded and staged according to UICC's 2002 guidelines. Determination of tumor origin was based on micro- and macroscopic evaluation of the resected specimen. Duodenal, cystic and neuro-endocrine tumors were excluded. Other exclusion criteria were: age>75, karnofsky-Index =/< 50, uncontrolled infection, previous chemo- or radiotherapy and aberrant vascular supply to the liver. Enrolled patients were prestratified for tumor origin (PDAC or NPPC). After recovery from surgery patients were randomized during their first visit to the outpatient clinic, according to a computer generated randomization list provided by the trial statistician. Treatment started within 6-12 weeks after surgery. Patients with complications resulting in prolonged hospital stay were not randomized. During or before the first CAI and before RT, all patients were restaged by Computed Tomography (CT). Follow-up consisted of clinical and laboratory examinations every 3 months. During the first two years CT was performed every 3 months and every 6 months afterwards. Clinical signs of recurrence were indications for additional imaging. All patients were monitored for 5 years or up to death. All survival data was cross-checked with the national population registry.

Adjuvant treatment

The treatment schedule was described in full detail previously[9]. Chemotherapy was administered through a catheter placed in the celiac trunk and left in place during the five treatment days of each cycle. Heparin was infused to prevent thrombosis. Cycles consisted of mitoxantrone on day one, followed by 5-fluorouracil/ folinic acid on days two to four and cisplatinum on day five.

Toxicity was monitored and the dose was reduced 20% in case of toxicity > World Health Organization (WHO) degree II. After two weeks radiotherapy was started. A total of 54Gy was delivered in a single dose of 1.8 Gy five days a week. CAI was continued afterwards up to a total of six cycles with an interval of four weeks between each cycle. Therapy was discontinued in case of serious toxicity, WHO III/IV.

Statistics

Primary outcome was overall survival. Secondary endpoints were toxicity and disease-free survival. With $\alpha = 0.05$ (two-sided) and $\beta = 0.20$, and pre-stratification by tumor origin, 125 patients had to be enrolled in each trial arm assuming a 2 year survival of 30% in the control groups and 50% in the experimental groups. Inclusion was stopped after 120 patients because gemcitabine based adjuvant therapy had become standard-care for PDAC and an observation-only group for PDAC was therefore considered unethical. Separate continuation of the trial for NPPC was not part of the protocol and therefore not considered.

Analyses were performed on an intention-to-treat basis. IBM SPSS Statistics version 20 for Windows was used for the current analyses. The Chi-square test was used for categorical and student-t for continuous variables. A p-value <0.05 (two-sided) was considered statistically significant. All p-values were rounded to three decimals. Survival was estimated using the Kaplan Meier method, significance calculated by log-rank test.

In addition, near-significant factors ($p< 0.100$) from the univariate analysis were entered in a multivariate cox-proportional hazards model, in accordance with the criteria for Proportional Hazards10. Hazard ratios are shown with 95% confidence interval (95%CI). Grade variables were considered of ordinal level and therefore coded as dummy variables.

Overall survival and disease-free survival

Primary analysis (whole group, n=120)

The primary analysis was based on 82 deaths. Longer follow-up led to registration of 18 more events. Therefore this survival analysis is based on 100 deaths. Long-term survival (defined as >60 months) was observed in 19 patients. Overall survival for patients suffering from PDAC is worse than survival for patients suffering from NPPC, regardless of therapy (Median survival 20 vs. 32 months, p<0.001). For the combined groups (whole study group) CAI/RT did not improve survival. Disease free survival was longer for patients treated by CAI/RT for the whole (PDAC and NPPC) study group, (13 vs. 8 months p=0.031). For each pre-stratified group results are very different:

Subgroup PDAC (n=62)

CAI/RT did not influence survival for PDAC (20 vs. 21 months p= 0.929). There was no significant effect of CAI/RT on time to progression in PDAC alone (12 vs. 8 months, p=0.214).

Subgroup NPPC (n=58)

For the NPPC group the number of long-term survivors after CAI/RT was twofold the number of long-term survivors after surgery alone (10 vs. 5). Although the survival curves (figure 1) clearly diverge, log-rank analysis did not reach statistical significance (median actual survival of 37 vs. 28 months (figure 1, p=0.077). However, Cox regression revealed a substantial effect of CAI/RT on survival, hazard ratio: 0.44, 95%CI: 0.23-0.83 p=0.011

Table 1, Summary of univariate and multivariate analysis.

Univariate and multivariate analysis: overall survival NPPC								
	No patients (%)		Univariate (log-rank)			Multivariate (Cox)		
	CAI/RT	Surgery only	Median	95% CI	p	HR	95% CI	p
Patients (N)	28	30						
Male	11	17	21	17-25				
Female	17	13	33	16-50	0.285			
PPPD	22	23	28	13-43				
Whipple	6	7	19	6-31	0.303			
Pathology:								
T2	9	8	33	11-55	Reference			
T3	13	16	28	10-47	0.435			
T4	6	6	18	8-28	0.025			
N0	12	12	36	23-49				
N1	16	18	21	17-25	0.037			
R0	23	29	25	13-36				
R1	5	1	20	0-51	0.081			
Differentiation grade:								
Well	4	5	60	-	Reference			
Moderate	17	19	24	7-36	0.022			
Poor	7	6	20	7-33	0.003	2.55	1.53-4.25	0.000
Surgery only		30	21	17-26				
Treatment CAI/RT	28		33	20-46	0.077	0.44	0.23-0.83	0.011

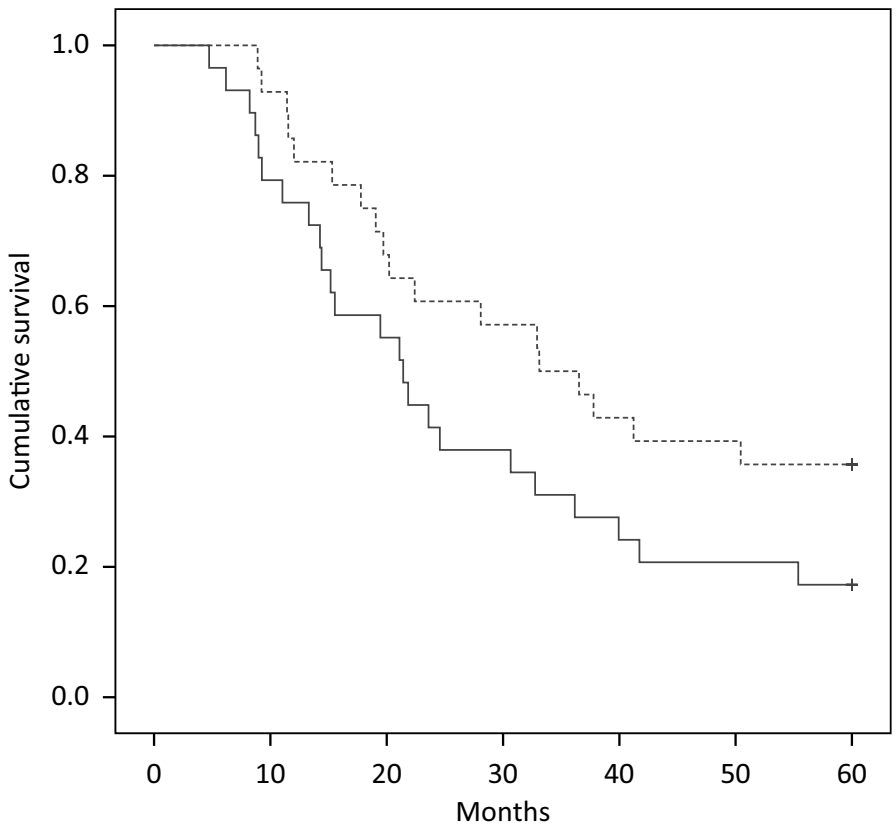
Table 2, NPPC, patients and cycles of CAI, Number of patients N(%), reasons for cessation of therapy are listed. Please note that more than half (15/28) of all patients received all 6 cycles.

CAI courses	0	1	2	3	4	5	6	Total
Patients N(%)	28(100)	25 (89)	23(82)	18(65)	17(61)	16 (57)	15(54)	28
Drop-out	3(11)	2(7)	5(18)	1(4)	1(4)	1(4)	0	13(46)
Progression	1(4)		2(7)					3(11)
Angio related			1(4)					1(4)
Toxicity		1(4)	2(7)	1(4)	1(4)	1(4)		6(21)
Patient factors	2(7)	1(4)						3(11)

The other independent factor was differentiation grade. Regardless of therapy, well-differentiated NPPC bare a favourable prognosis compared to moderately and poorly differentiated NPPC (Figure 3). A detailed description of univariate and multivariate analysis for overall survival is shown in Table 1. Factors were tested for their independent contribution in the model. For NPPC time to progression appears to be longer after adjuvant CAI/RT (Figure 2, 19 vs. 8 months, p=0.103 log-rank, Hazard ratio for recurrent disease 0.48 95% CI: 0.25 to 0.90 p= 0.022). The other independent factor in the same regression model was differentiation grade.

The recurrence pattern is shown in Table 3. Interestingly, CAI/RT effectively suppressed the long-term occurrence of liver metastases in patients with NPPC, from 17 to 8 cases (p = 0.038 Chi-square, hazard ratio 3.27 95% CI: 1.10 to 9.80). No effect could be shown in time to occurrence of liver metastasis.

Figure 1, Overall survival, Kaplan Meier, NPPC: CAI/RT (dotted line) vs Surgery only, p = 0.077 log-rank, hazard ratio: 0.44 95%CI: 0.23-0.83 p=0.011, Below figure: patients at risk

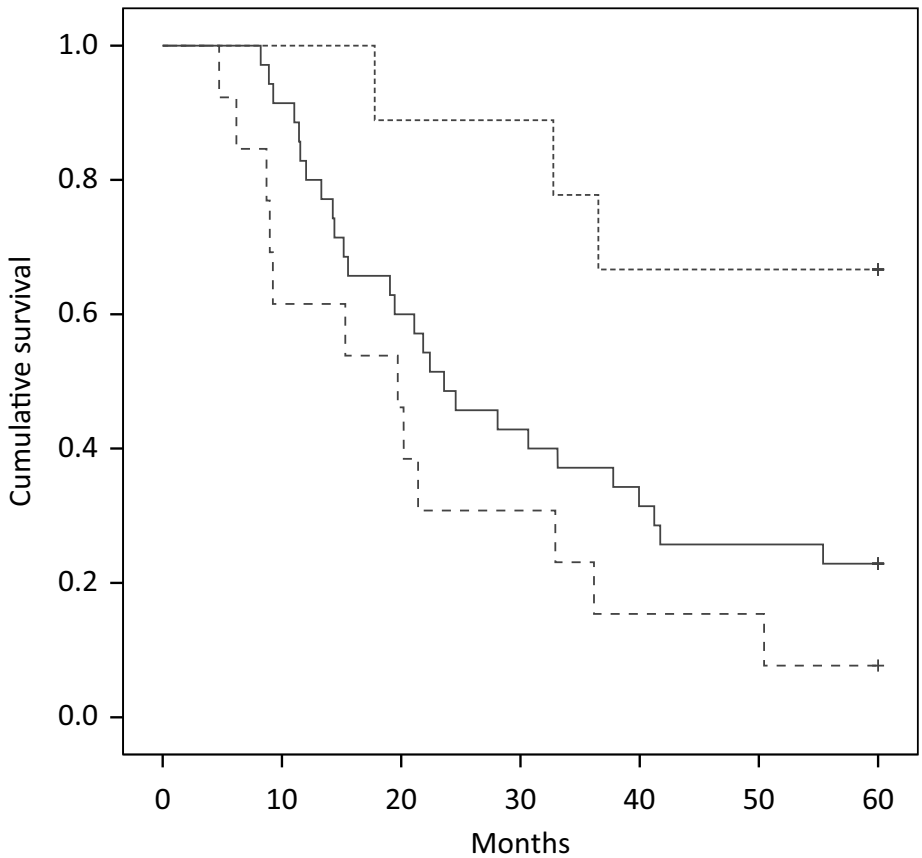


Months	0	10	20	30	40	50	60
CAI/RT	28	27	20	17	13	12	10
Surgery only	30	23	17	12	8	7	5

Table 3, Recurrence pattern, PDAC: pancreatic cancer, NPPC: non-pancreatic periampullary cancer, cumulative occurrence of livermetastases is significantly less frequent after CAI/RT than surgery alone for NPPC (8 vs. 17 patients, p=0.038), this effect was not observed in PDAC. We observed no effect on local recurrence in either NPPC or PDAC.

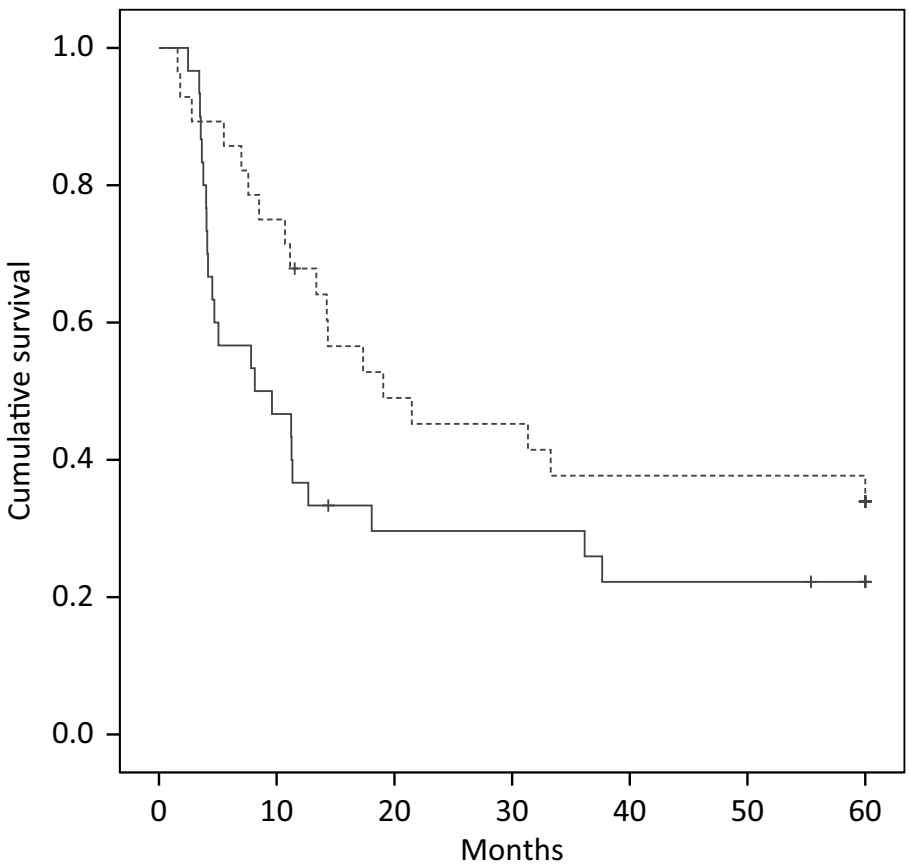
Recurrence pattern	PDAC					NPPC			
	CAI/RT	%	Control	%	P	CAI/RT	%	Control	%
N	31		31			28		30	
First Recurrence									
Local	14	45%	19	61%	0.309	11	39%	9	30%
Liver	14	45%	11	35%	0.605	7	25%	13	43%
Local and Liver	6	19%	4	13%	0.731	2	7%	3	10%
Lung	5	16%	3	10%	0.707	3	11%	3	10%
Other	0	0%	6	19%	0.024	3	11%	3	10%
Cumulative Recurrence									
Local	15	48%	22	71%	0.120	13	46%	12	40%
Liver	15	48%	16	52%	1.000	8	29%	17	57%
Local and Liver	7	23%	11	35%	0.402	4	14%	7	23%
Lung	5	16%	3	10%	1.000	3	11%	5	17%
Other	1	3%	9	29%	0.012	5	18%	5	17%

Figure 2, Disease-free Survival, NPPC, Kaplan Meier, CAI/RT (dotted line) vs. Surgery only, (19 vs. 8months, p = 0.103 log-rank, Hazard ratio for recurrent disease 0.48 95% CI: 0.25 to 0.90 p = 0.022). Below figure: patients at risk.



Months	0	10	20	30	40	50	60
CAI/RT	28	21	13	12	10	10	10
Surgery only	30	14	8	8	6	5	5

Figure 3, Kaplan Meier, Overall survival, according to differentiation grade, top to bottom line: Well, moderate and poor differentiated NPPC.(Well vs Poor p<0.01, Well vs Moderate p=0.022, Moderate vs Poor p=0.122) Below figure: patients at risk



Months	0	10	20	30	40	50	60
Well	9	8	7	7	5	5	5
Moderate	36	33	22	14	12	10	9
Poor	13	8	6	2	2	2	1

Discussion

This is a detailed long-term outcome analysis of a randomized clinical trial comparing survival after adjuvant therapy for PDAC and NPPC with observation alone. We confirm the results of the original report that CAI/RT does not improve survival for PDAC. The effect of CAI/RT in this group is disappointing and confirms that true pancreatic cancer has a dismal prognosis despite the addition of chemotherapy and radiotherapy. However, for NPPC survival appears better in the CAI/RT group. CAI/RT also effectively reduced the occurrence of liver metastases. This effect was sustained throughout the long-term follow-up.

Our results confirm that NPPCs are probably a separate family of tumors with a different biological behavior. This hypothesis is supported by the superior survival of NPPCs, even after adjusting for tumor size, positive lymph nodes and stage[11,12]. Evidence is mounting that these tumors may have a different genetic basis and express different proteins, microRNA and growthfactor receptors[12-16]. Perhaps in the future we can use these biomarkers to identify more specific subtypes of NPPC and PDAC and select a more targeted type of adjuvant therapy.

In pursuit of improvement of survival after surgery for pancreatic cancer, several randomized trials have been conducted offering adjuvant chemotherapy both with and without concomitant radiotherapy. This has led to the consensus that gemcitabine based adjuvant chemotherapy improves outcomes after surgery for PDAC[17]. The role of adjuvant radiotherapy remains doubtful[18].

On the other hand, the benefit of adjuvant chemotherapy for patients with NPPC remains largely unclear. The first trial was the well-known EORTC trial, offering a 5-fluorouracil based regimen with concomitant radiotherapy, but without an effect on survival[3,8]. In the EORTC trial adjuvant 5-fluorouracil based chemotherapy with concomitant radiotherapy (40Gy) was compared to surgery alone. Liver metastases occurred in 50% of the patients. There were no significant differences in occurrence of these liver metastases between treatment groups. Neoptolemos et al.[4] recently published the only other recent randomized study on adjuvant therapy for NPPC. This trial compared three study groups; 5-fluorouracil based chemotherapy, gemcitabine based chemotherapy and surgery only. The authors

were unable to demonstrate improved survival after gemcitabine or 5-fluorouracil based adjuvant chemotherapy in the primary analysis of aforementioned groups. However in a multivariate analysis, after adjusting variables of age, bile duct cancer, poor tumor differentiation, and positive lymph nodes the authors observed a modest benefit for adjuvant chemotherapy. This study clearly shows that the common adjuvant schedules for PDAC cannot be extrapolated to the treatment of NPPC. In the recent ABC-2 trial[19], gemcitabine was combined with cisplatinum, and led to a survival benefit for patients suffering from advanced cholangiocarcinomas. The patients included in this study suffered from a range of intrahepatic, extrahepatic and metastastic bile duct cancers. Perhaps the addition of cisplatinum may have been crucial for the response of ampullary and distal bile duct cancers (NPPC) to CAI in our study.

The rationale for CAI/RT in the present study was twofold: reduction of liver metastases by CAI and improved local control by RT. And indeed, for NPPC CAI/RT led to reduction of occurrence of liver metastases and a substantial effect was observed on median survival, disease-free survival and also more long-term survivors. Interestingly, we could not demonstrate an effect on local recurrence. The EORTC study also included RT, 40Gy compared to the 54Gy in the present study. The EORTC trial also did not show any effect on local recurrence. Therefore it is more likely that the CAI is responsible for fewer liver metastases and consequently a positive effect on survival and not RT. Two phase II clinical trials and a case study preceded our trial[5,20,21]. Both trials showed a decrease in occurrence of liver metastases and improved survival after CAI. The underlying principle is that by infusing selectively, a much higher dose can be achieved in the target organ, in this case the liver. It is not precluded that for pancreatic cancers, the mitoxantrone, 5-flourouracil and cisplatinum combination used in this study may be inferior to a gemcitabine based regimen. Furthermore, recent developments of new therapeutic agents and combination therapy have led to more effective systemic therapy in metastatic pancreatic cancer using a 5-fluorouracil, leucovorin, irinotecan en oxaliplatin (Folfirinox) regimen[22]. We speculate that a different agent or combination could be effective as CAI in PDACand that we should not completely discard the CAI concept as a possible means of delivering other more effective chemotherapeutics to patients suffering from PDAC.

In the Cox regression analysis we showed that both differentiation grade and adjuvant treatment were of independent influence on overall and disease-free survival in NPPC. Differentiation grade was inversely correlated with survival (Figure 3), which is concordant with other studies[4]. Survival after resection of well- differentiated cancers is much better compared to moderately/poorly differentiated tumors. It is questionable whether well-differentiated tumors should be treated with this adjuvant regimen.

This long-term analysis shows interesting effects of adjuvant CAI/RT on survival in patients with NPPC that were not revealed in the primary analyses. Although this group is relatively small (N=58), the trial was stopped preliminary and a benefit was only observed in this pre-stratified group we provide some evidence that this concept of CAI/RT may be beneficial to these patients. We acknowledge that this study is underpowered. Although a small group size would rather lead to a type two error (i.e. no effect of therapy in the analyses while a true effect might have been present) than overestimation of the effect of CAI/RT. Finding a positive effect in a pre-stratified group advocates further studying of this concept in patients with NPPC, in particular in those with moderately or poorly differentiated tumors. In addition, this is the only study to date that shows any substantial beneficial effect of adjuvant therapy for this particular group of patients.

Although this schedule of CAI/RT is intense, it did not adversely affect quality of life during the short time some of the patients live after “curative” resection[23]. Toxicity was relatively mild. Delivering CAI to a large number of patients is a logistic challenge and requires training of medical staff and dedicated nurses. Further developments of minimally invasive isolated perfusion devices may prove to be more practical in use and more effective in delivering chemotherapy to the liver alone, were the effect of our regimen was most noticeable.

In conclusion, patients with resectable NPPC may benefit from adjuvant CAI as it has a substantial effect on overall and disease-free survival and as it effectively and enduringly reduces the occurrence of liver metastases. The value of RT for local control remains doubtful. The results of this trial warrant further investigation by means of a dedicated trial on adjuvant CAI for NPPC. This trial should enroll patients in three treatment arms: 1. surgery only (control), 2. systemic gemcitabine + cisplatinum (based on the ABC trial) and 3. gemcitabine + cisplatinum based CAI.

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Chapter 5

Long-Term Survival after Radical Resection for Pancreatic Head and Periampullary Cancer: A Potential Role for the EGF-R

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Digestive Surgery, 2007;24(1):38-45

Abstract

Background/Aim:
Pancreatic cancer has a dismal prognosis. Ampullary cancer (defined as cancer of the ampulla of Vater or the distal common bile duct) has a better prognosis and is thought to be a biologically different tumor. The aim of this study was to find factors that could predict survival after radical (R-0) resection for pancreatic head and ampullary cancers.

Methods:
We analyzed clinical and pathological data from 93 patients who underwent a true R-0 resection for pancreatic head or ampullary cancer. Furthermore, we performed a tissue microarray protein expression analysis for several growth factor receptors and oncogenes: HER-2, EGF-R, ER, PR, C-myc, p53, p16, RB-1, and chromogranin A as a neuroendocrine differentiation marker.

Results:
Median survival (14 vs. 42 months) and time to recurrence (16 vs. 42 months) were significantly longer for ampullary than for pancreatic head cancers. Preoperative pain, perineural invasion, lymph node metastasis, and tumor differentiation grade are indicators of a poor survival. No differences in protein expression were found between groups, except for EGF-R which was expressed more in pancreatic head cancers ($p = 0.026$).

Conclusions:
Outcomes for ampullary cancers are better than for pancreatic head cancers. This different biological behavior can possibly be explained by differences in EGF-R expression.

Introduction

Of all gastrointestinal malignancies, pancreatic cancer has the poorest prognosis, with a 5-year survival of less than 5%[1,2]. Men are more frequently affected by this disease than women (relative risk 1.5)[2]. Ampullary cancers, however, carry a better prognosis[3]. This evident difference may be due to earlier clinical presentation or different biological behavior. Curative resection offers the only chance of cure, but is possible in only 10% of the patients[4]. Adjuvant chemotherapy and chemoradiotherapy after curative resection are of limited value[5]. Even after a macroscopically radical resection (R-0), distant micrometastases probably already exist[6], and tumor cells are often observed at one or more edges of the resected specimen (R-1)[7,8]. Reported relevant prognostic variables for survival after resection are: tumor size, lymph node metastasis, histological differentiation, and resection status[9-12]. Most studies include R-0 as well as nonradical (R-1/R-2) resections. To eliminate the apparent effect of R-1 status on (local) recurrence and survival, it would be interesting to evaluate true R-0 resections only.

The aim of this study was to determine clinical, histological, and molecular factors that could predict recurrence and survival after R-0 resection. Special focus is on the differences between ampullary (defined as cancer of the ampulla of Vater or distal common bile duct) and pancreatic head cancers. Additionally a tissue microarray (TMA) analysis of resected tumors (R-0) was added in order to search for relevant molecular factors[13,14]. For immunohistochemical staining, we selected common antibodies raised against proteins that are frequently expressed in (pancreatic and ampullary) cancers such as: retinoblastoma (RB-1)[15,16], p16[17], C-myc[18], and p53[17]. Potential targets for therapy: HER-2[19] and epithelial growth factor receptor (EGF-R)[20]. Potential markers for a male/female difference: estrogen receptor (ER) and progesterone receptor (PR)[21]. Chromogranin A was used as a neuroendocrine differentiation marker.

Materials and Methods

From a consecutive series of 176 resections for pancreatic head and ampullary adenocarcinomas, specimens were revisited. For the purpose of this study we defined ampullary cancer as cancer of ampulla of Vater and distal common bile duct. Duodenal tumors were not included. Pancreatic cancers adjacent or close to the ampulla were classified as pancreatic head cancer. R-0 was defined as absence of macroscopic and microscopic residual tumor >1 mm from the margin of the resection specimen. Even if there were lymph node metastases restricted to the resected area, the resection was considered R-0. Seventy patients had an R-1 and 6 patients an R-2 resection. In 7 cases the pathologist was not able to define whether a true R-0 resectionwas performed. Therefore, 93 patients were evaluated in this study.

One pathologist reviewed all specimens of R-0 resected tumors. Histological differentiation, tumor size (T), location and extent, as well as vasoinvasive growth (small blood vessels), perineural invasive growth, and lymph node metastasis (N) were assessed. Staging was performed according to the UICC classification, 2002[22]. Preoperative data, including age, gender, weight loss, pain (back pain and pain in the epigastric region), jaundice, and diabetes mellitus, were obtained from the clinical records.

Preoperative Staging and Surgical Techniques

All patients underwent conventional ultrasonography and/or computed tomographic scanning. Most patients (76%) underwent endoscopic retrograde cholangiopancreatography and subsequent preoperative biliary drainage using endoprostheses. After excluding extraregional and distant metastases, an estimation of the resectability was made by judging the involvement of common hepatic artery and superior mesenteric vein and portal vein. Dissection of the pancreas was to the left of superior mesenteric vein/portal vein. Histological examination of the frozen section of the remaining pancreatic surface was performed. In case of a standard Whipple (SW) resection, one third of the stomach was also resected. Only tumors without macroscopic infiltration of the

postpyloric duodenum and in absence of positive lymph nodes along the pylorus were treated by a pylorus-preserving pancreaticoduodenectomy (PPPD). Lymph nodes were dissected on the right-hand side of superior mesenteric vein/superior mesenteric artery up to the celiac trunk and in the hepatoduodenal ligament along the common hepatic artery. Perioperative data included type of operation performed (PPPD or SW), blood loss (ml), and transfusions (units). Postoperative data included complications, hospital stay, and adjuvant therapy (5-FU and radiotherapy).

Tissue Microarray

We identified 75 patients for whom paraffin blocks were available. In all other cases paraffin blocks were either lost or insufficient. The TMA was constructed as described by Kononen et al.[13] For each carcinoma, we prepared three tissue cores of 0.6 mm in diameter from the paraffin tissue block to ensure adequate representation of the neoplastic cells. The tissue cores from each carcinoma were then mounted in linear arrays in a paraffin TMA block. Tissue cores from various organs (pancreas, duodenum, and gallbladder) were used as controls, for orientation purposes, and to estimate background labeling for each of the immunohistochemical markers. Immunohistochemistry labeling was performed according to standard protocols. In brief, 4-μm sections for each TMA were transferred to StarFrost® slides (Starfrost, Berlin, Germany), and immunostaining was performed using the UltraVision Large Volume Detection System Anti-Polyvalent, HRP (Lab Vision, Fremont, Calif., USA) after deparaffinization. Microwave (700 W) pretreatment was performed for 15 min using citrate buffer (100 mM citric monohydrate, pH 6.0). Antibodies (clone) used for immunostaining were ER-α (1D5), PR (PgR636), p16 (E6H4), p53 (DO-7), RB-1 (RB-1), EGF-R (H11), and HER2 (C-erB-2) (DAKO, Glostrup, Denmark); chromogranin A (LK2H10) (Biogenex, San Ramon, Calif., USA), and C-myc (9E10) (Santa Cruz Biotechnology, Santa Cruz, Calif., USA).

TMA Scoring Strategy

A pathologist and an experienced analyst examined the TMA slides for each immunohistochemical marker with a multiobserver microscope. Both observers were blinded to the type of cancer and its location on the slide. Only moderate or strong labeling was scored as positive. Weak or 'blush' labeling was ignored. A two-tiered scheme (positive or negative) was used for scoring the TMA. A proportion exceeding 1% of positive cells was regarded as protein overexpression. A negative or positive score for each carcinoma was determined after examining the three tissue cores. Focal and diffuse positive scores were combined for the sake of statistical analysis.

Statistics

Survival analysis consisted of overall survival and time to recurrence. Curves were calculated by the Kaplan-Meier method, followed by log-rank tests. Factors, which showed to be significant in the univariate analysis, were entered into a multivariate Cox proportional hazards model to evaluate their independent prognostic value by backward elimination. To determine whether factors differed between patients with ampullary versus pancreatic head carcinomas, appropriate interaction terms were used.p (two-sided) < 0.05 was considered statistically significant. All calculations were performed using SPSS version 13.0 (SPSS, Chicago, Ill., USA).

Results

Survival

The follow-up period was more than 10 (median 12) years. Median survival and time to recurrence were significantly longer for ampullary than for pancreatic head cancers (14 and 42 months, p = 0.001, and 16 and 34 months, p = 0.024, respectively; table 1). Survival curves are shown in figure 1. There were no differences between groups with regard to site of recurrence (p = 0.497).

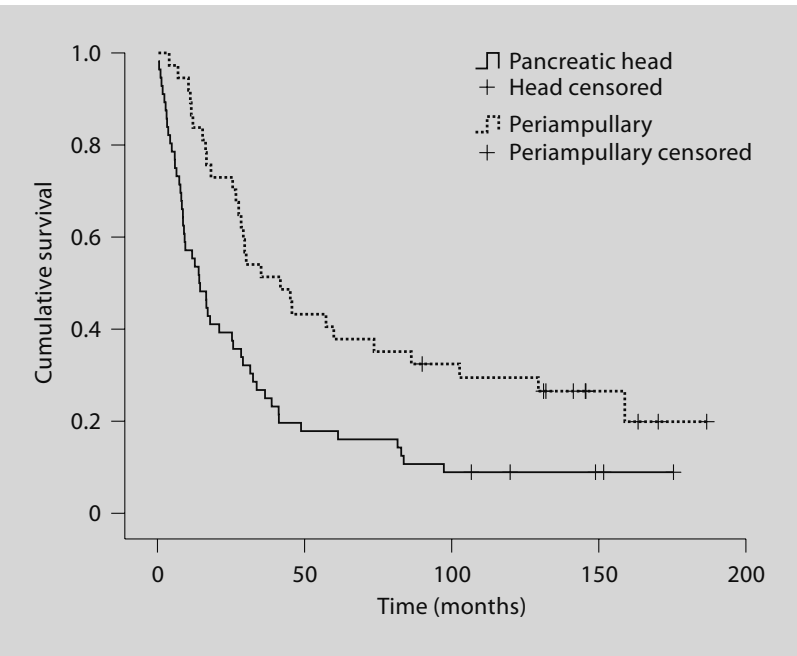

Fig. 1. Kaplan-Meier survival curves for ampullary and pancreatic head cancers (p = 0.001, log-rank test).

At risk	Time (months)							
	12	24	36	48	60	72	84	120
Pancreatic head (n = 56)	31	22	15	11	10	10	5	3
Ampullary (n = 37)	32	27	19	16	14	13	13	10

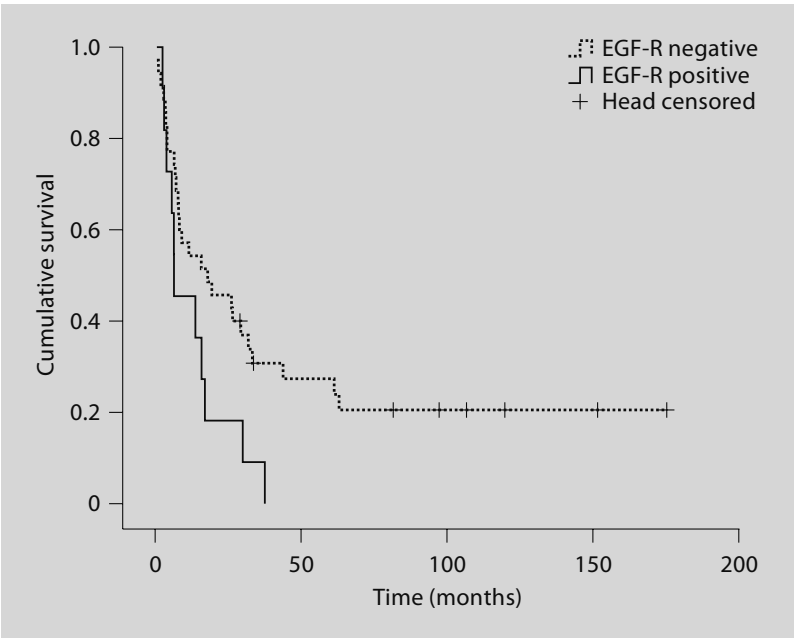

Fig. 2. Kaplan-Meier curves for EGF-R expression in pancreatic head cancers, time to recurrence (p = 0.037, log-rank test).

At risk		Time (months)							
		12	24	36	48	60	72	84	120
Positive	(n = 11)	5	2	1	0				
Negative	(n = 35)	19	16	9	8	8	6	4	2

Preoperative Factors

No differences in preoperative factors were observed between both groups. Survival was better for women than for men in the pancreatic head cancer group, but not in the ampullary cancer group. Preoperative abdominal and back pain significantly influenced survival and time to recurrence for both groups. Jaundice was a predictor of survival and time to recurrence in ampullary cancers. Weight loss and diabetes mellitus did not influence survival (tables 1, 2).

Perioperative Findings

The surgical technique used (PPPD vs. SW) did not correlate with survival (p = 1.000). Blood losses were higher and blood transfusions (units) more often required in the pancreatic head cancer group (p = 0.028 and 0.038). Hospitalization was longer for pancreatic head cancers (p = 0.048). However, these factors did not influence survival (tables 1, 2).

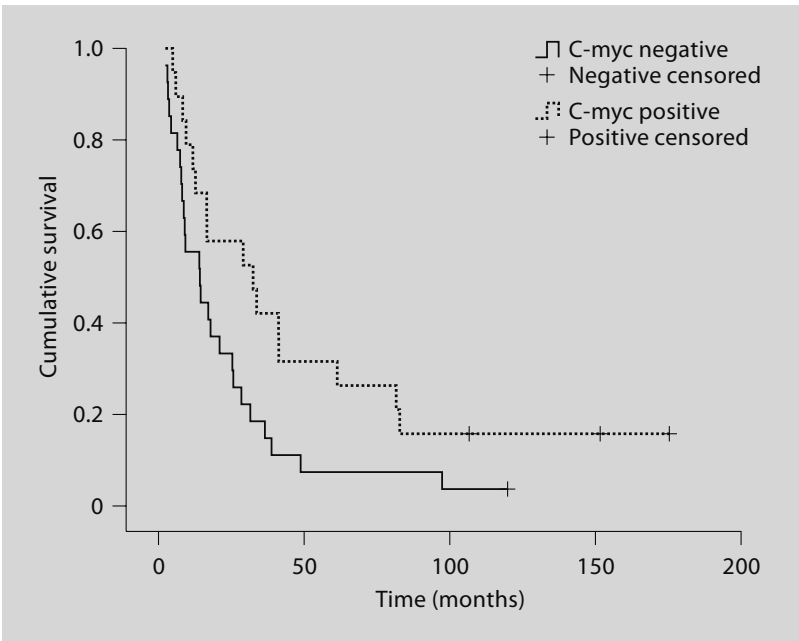

Fig. 3. Kaplan-Meier survival curves for C-myc expression in pancreatic head cancers (p = 0.034, log-rank test).

At risk		Time (months)							
		12	24	36	48	60	72	84	120
Positive	(n = 19)	14	10	7	5	5	4	2	2
Negative	(n = 27)	15	9	5	3	2	2	2	0

Mortality and Morbidity

Two patients (2%) died postoperatively because of aspiration pneumonia and sepsis. These patients remained in our analysis because of the intent-to-treat principle. Postoperative complications occurred in 44 cases (48%). In 5 patients (5%), leakage of the pancreaticojejunostomy was seen, defined as the presence of amylase in drainage or abdominal fluid (>3× serum concentration). Six patients developed a pancreaticocutaneous fistula. Leakage of the biliary anastomosis occurred in 1 patient. Eleven patients (12%) had an intra-abdominal abscess. Minor complications were found in 17 patients (18%). Complications did not influence survival or time to recurrence (tables 1, 2).

Adjuvant Therapy

Adjuvant therapy (5-FU and radiotherapy) was given to 28 patients, distributed evenly between both groups ($p = 0.363$; table 1). Survival data are omitted because of bias. These patients participated in a trial, in which we compared the effects of chemoradiation with those of surgery alone[3].

Pathological Factors

Pancreatic head tumors were larger ($p = 0.001$) than ampullary tumors. Perineural invasion was also significantly more often observed in this group ($p = 0.001$).

The strongest negative histological factor for survival and time to recurrence was lymph node involvement in the resected specimen. Tumor diameter as well as extent of the tumor did not influence survival. Histological grading was correlated with survival and time to recurrence for pancreatic head cancers. Invasive growth into intrapancreatic perineural tissue significantly influenced survival ($p = 0.001$) and time to recurrence ($p = 0.000$) in ampullary cancers. Tumor invasion of small surrounding blood vessels was not correlated with survival (tables 1, 2).

Table 1. Group comparisons

	Factor positive (%)			p (Fisher's exact t test)
	head (n = 56)	ampullary (n = 37)	total (n = 93)	
Preoperative factors				
Male sex	37 (66)	19 (51)	37 (39)	0.191
Age, years	63	63	63	0.993
Pain	31 (55)	20 (56)	51 (55)	1
Jaundice	49 (88)	30 (83)	79 (86)	0.76
Pruritus	13 (23)	15 (44)	28 (31)	0.059
Weight loss	45 (80)	28 (78)	73 (79)	0.796
>5 kg	23 (51)	18 (64)	41 (56)	0.335
Obstruction	5 (9)	5 (14)	10 (11)	0.505
Diabetes	10 (18)	8 (22)	18 (20)	0.603
Endoprosthesis	44 (79)	27 (71)	71 (78)	1
Perioperative factors				
PPPD	28 (50)	18 (49)	46 (50)	1
PV resection	1 (2)	0	1 (1)	1
SMA resection	0	1 (3)	1 (1)	0.398
Blood loss, ml	2,700	1,950	2,400	0.028[a]
>1 liter	5 (9)	6 (18)	11 (13)	0.324
Transfusions, U	2.4	1.5	1.2	0.038[a]
Transfused patients	22 (41)	7 (21)	29 (33)	0.064
Complications	24 (44)	20 (54)	44 (48)	0.396
Leakage pancreaticojejunostomy			5 (5)	
Pancreatic fistula			6 (6)	
Leakage biliary anastomosis			1 (1)	
Abdominal abscess			11 (12)	
Minor			17 (18)	
Mortality			2 (2)	
Hospitalization, months	28	21		0.048[a]
Radiation/5-Fu	19 (34)	9 (24)	28 (30)	0.363

Table 1 (continued)

	Factor positive (%)			p (Fisher's exact t test)
	head (n = 56)	ampullary (n = 37)	total (n = 93)	
Pathological factors				
T1	7 (12)	8 (22)		not tested
T2	15 (27)	16 (43)		not tested
T3	34 (61)	13 (35)		not tested
Diameter <2 cm	23 (41)	28 (76)	51 (55)	0.001
N	29 (52)	26 (70)	55 (59.1)	0.088
M	0	0	0	
G1	6 (11)	7 (19)		0.509[b]
G2	43 (79)	25 (68)		
G3	7 (12)	5 (13)		
R-0 resection	56 (100)	37 (100)		
Perineural invasion	31 (55)	7 (19)	38 (41)	0.001
Vasoinvasive	11 (20)	5 (14)	16 (17)	0.579
Survival				
Recurrences	41 (73)	26 (70)	67 (72)	0.816
Local	7 (18)	6 (26)		0.497[b]
Distant	18 (46)	8 (35)		
Both	12 (31)	9 (39)		
Deaths	51 (91)	28 (76)	79 (85)	0.073
Median survival, months	142	42		0.001[c]
Median time to recurrence, months	16	34		0.024[c]

PPPD = Pylorus-preserving pancreaticoduodenectomy; PV = portal vein;SMA= superior mesenteric artery; T = primary tumor; N = positive lymph nodes; M = metastasis; G = differentiation grade. a t test; b chi-square for 3 x 2 tables; c log-rank test.

Tissue Microarray

The overall failure rate secondary to lack of interpretable neoplastic tissue was 3%. In all other cases in whom cores were without malignant glands, interpretation of the labeling pattern was possible with the remaining tissue cores from the same carcinoma. Although histologically very similar, adenocarcinomas of the ampullary region are thought to be biologically different from pancreatic ductal adenocarcinomas. However, for most tested proteins, the labeling was not significantly different between both groups. Interestingly, EGF-R is expressed more frequently in pancreatic head than in ampullary cancers (p = 0.026). For pancreatic head cancers, EGF-R overexpression is an indicator for a shorter time to recurrence (p = 0.037; fig. 2). A trend was observed towards a poor survival (median 14 vs. 25 months; p = 0.084). Furthermore, no long-term survivors were observed in the EGF-R-positive group. C-myc overexpression is correlated with improved survival (p = 0.034; fig. 3) and longer time to recurrence (p = 0.015). It is clear that these last two factors are of significance for pancreatic head cancers, but not for ampullary cancers (tables 1, 2).

Table 2. Univariate analysis

Factor positive	Direction of effect	Relation to survival			Time to recurrence		
		head (n = 56)	ampullary (n = 37)	pooled[a] (n = 93)	head (n = 56)	ampullary (n = 37)	pooled[a] (n = 93)
Male sex	–	0.028	0.688	0.046	0.072	0.614	0.086
Pain	–	0.018	0.001	0.000	0.006	0.000	0.000
Jaundice	+	0.364	0.029	0.044	0.773	0.017	0.097
N	–	0.064	0.013	0.004	0.016	0.006	0.000
G	–	0.034	0.624	0.012	0.046	0.384	0.012
Perineural invasion	–	0.273	0.001	0.025	0.053	0.000	0.001
C-myc	+	0.034	0.968	0.078	0.015	0.858	0.038
EGF-R	–	0.084	not tested	0.080	0.037	not tested	0.042

Pain = Preoperative back pain and abdominal pain; N = positive lymph nodes; G = differentiation grade.
[a] Test for trend.

Multivariate Analysis

All factors that were significant in the univariate analysis were entered into a Cox regression model. Independent prognostic factors for survival for the pancreatic head group were: preoperative back pain and abdominal pain (B(exp): 0.548/p = 0.05) and differentiation grade (2.004/0.016). No independent prognostic factors were found for ampullary cancers.
For time to recurrence independent prognostic factors for the pancreatic head group were: preoperative back pain and abdominal pain (0.446/0.015), positive lymph nodes (0.491/0.033), and EGF-R overexpression (0.448/0.034). Independent prognostic factors for ampullary cancers were preoperative back pain and abdominal pain (0.224/0.007) and perineural invasion (0.199/0.031).

Discussion

Median survival and time to recurrence after R-0 resection were significantly longer for ampullary than for pancreatic head cancers. This evident difference may be due to earlier clinical presentation or different biological behavior. We found no differences in preoperative factors between both groups.

Preoperative back pain and abdominal pain was found in 51 patients (55%). It was also an independent negative prognostic factor for time to recurrence for both groups and for survival in univariate analysis. We further found that intrapancreatic perineural invasion was a negative prognostic factor for survival (not for pancreatic head cancers, p = 0.273) and time to recurrence in the univariate analysis (both groups). Preoperative pain as a negative prognostic factor has been reported earlier by Ridder and Klempnauer[23] and Okusaka et al.[24] Forty-one percent of the patients with preoperative pain had intrapancreatic perineural growth, and 55% of the patients with perineural growth had preoperative pain. These findings suggest that pain is not always caused by intrapancreatic perineural growth and vice versa.

Indeed, pain is usually interpreted as resulting from tumor infiltration into extrapancreatic (retropancreatic) splanchnic nerves and thus may indicate advanced tumor growth beyond the borders of the pancreas.

In the present study, 72% of all patients had recurrence of their cancer, and almost half of all recurrences were local (table 1). This is similar to previously reported rates in two large trials on adjuvant therapy done by Klinkenbijl et al.[3] and Neoptolemos et al.[25] Although both studies included only 18–22% R-1 resections, the reported rate of recurrence was approximately 70%, and 37–52% of these recurrences were local.

Possibly our definition of R-0 resection may have included some 'irradical' resections. Verbeke et al.[26] suggested that R-1 resection can be underestimated, when the pathological examination is not completely standardized. These authors compared pathological examinations for two consecutive periods with and without a highly standardized protocol. The number of R-0 resections for pancreatic cancer during the standardized period was lower than during the nonstandardized period ($p = 0.009$). Interestingly, long-term survival for pancreatic cancer was not predicted by resection status in either cohort. A similar observation was made by Neoptolemos et al.[12,25] in a subanalysis of their trial on adjuvant therapy. They found that the resection status was not an independent prognostic factor for survival. Only after omission of nodal status and differentiation grade, the resection status became significant[12,25].

It is intuitive that R-1 resection is a predictor of a poor survival, and indeed long-term survivors are sparse after R-1 resection. Therefore, R-0 resection is often considered to be the most important factor predicting a favorable outcome. Consequently, extended resections, including extensive clearing of retroperitoneal soft tissue and lymphatic tissue, have been advocated. Several randomized controlled trials[27-29] were performed, but failed to show a survival benefit for extended surgery. In a recent study performed by Hishinuma et al.[30, 27] patients who had undergone extended resection were studied at autopsy. Most resections were R-0 (25/27), 3 patients died postoperatively, the majority died of metastatic disease, and only 4 patients died due to local recurrence. Evidence of local

recurrence was found in 18 of 24 patients (75%). This study clearly shows that even after extended surgery, local recurrences are still frequent. Possibly R-0 and R-1 resections are both accompanied by (occult) metastatic disease. Indeed, in an overview of advanced molecular detection techniques[6], it was shown that tumor cells can be found pre- and perioperatively in peritoneal lavage fluids, liver, blood, 'tumor-negative' lymph nodes, and bone marrow of patients without 'conventional' evidence of metastatic disease. As a result, R-0 resection offers an opportunity for long-term survival for a limited number of patients. However, in the majority of the cases, resection cannot provide cure, and recurrence remains imminent. This further emphasizes the importance of additional prognostic parameters to predict outcomes after 'R-0' ('R < 1') resection and to select patients in need of (aggressive) systemic adjuvant therapy.

The TMA technique proved to be a simple, efficient, and relatively inexpensive method to explore the protein expression in large groups of patients. Our failure rate of 3% is acceptable and did not cause statistical problems. The expression of p53 and p16 proteins is well known in pancreatic cancer[17]. Nevertheless, their prognostic value remains unclear. We found a similar expression of p16 (21–33%) (table 1) to that reported in previous studies (13–59%)[17]. The expression of p53 in our study was lower (19–20%) than previously reported (35–69%)[17]. This may be due to our techniques or a selection bias in our group (R-0). RB-1 protein expression has been reported previously in tissue and cell lines, the exact prevalence, however, was unclear[31,32]. We found RB-1 to be positive in 62–77% of the cases. C-myc, a proto-oncogene, expression has been previously reported[18]. We found improved survival for patients with overexpression of this protein. This is counterintuitive, since, as a known proto-oncogene, it is thought to promote oncogenesis and tumor growth. Alternatively, C-myc expression is thought to be an early step in the rapid oncogenesis of pancreatic cancer[33] and may be an indicator of a relatively early point in tumor progression. Hypothetically, this implicates an early-stage disease and thus a less aggressive nature. Nevertheless, the exact role of C-myc remains unclear. We found 22% of the cancers to be positive for chromogranin A, a neuroendocrine differentiation marker. This is similar to the previous report on neuroendocrine differentiation by Tezel et al.[34] However, these authors did not find any chromogranin A-positive

tumors in their specimens, in contrast to 18–36% expression of other neuroendocrine markers (NCAM, NSE, synaptophysin, CD57). This may be due to differences in antigen retrieval, immunohistochemical techniques, and/or antibody clones used. HER-2 was positive in only 9–11% of the cases and did not show a correlation with survival. This is comparable to previously published results (8–58%)[17].

EGF-R, a well-known growth factor receptor, was positive in ampullary cancers (4%), but significantly more often in pancreatic head cancers (24%; p = 0.026). This is in accordance with expression rates reported previously (28–68%) [17]. The difference in EGF-R expression between pancreatic head and ampullary cancers was previously suggested by Friess et al.[35] Although their study did not provide matched clinical and survival data, they suggested a role for the EGF-R in the less favorable outcomes of pancreatic cancers. In our study, EGF-R overexpression was a negative prognostic factor for time to recurrence (p = 0.037) and possibly survival (p = 0.084). This effect on survival has been described previously in several studies, while others found no effect[17,36]. For head and neck cancer, the EGF-R expression was shown to be correlated with survival and relapse[37]. In breast and colorectal cancers, however, no correlation was evident[38,39].

In conclusion, the differences in biological behavior between pancreatic head and ampullary cancers could be confirmed in this study. The poor prognosis of patients with pancreatic head cancer can possibly be related to the increased EGF-R expression of these tumors as compared with ampullary cancers. Our data support the rationale to use drugs that have recently been designed to target the EGF-R selectively (Tarceva, Iressa, Erbitux) in adjuvant therapy regimens[40].

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Chapter 6

Distiction between Pancreatic and Non Pancreatic Periampullary Cancers by Comparative Genomic Hybridization

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Abstract

Introduction

Resectable pancreatic adenocarcinoma (PDAC) and non-pancreatic periampullary cancers (distal common bile duct, ampulla of Vater: NPPC) require similar surgical treatment, but a different (neo-) adjuvant chemotherapeutic approach. Aim of this study was to find genetic differences between PDAC and NPPC, which could be used in pre-operative differentiation to guide therapy.

Methods

From our archives we selected 50 paraffin embedded samples of resected PDAC and NPPC (25/25). After isolation of DNA, samples were analyzed by means of array based Comparative Genomic Hybridization (aCGH).

Results

Both groups had comparable baseline characteristics. Survival was superior for NPPC, even after correction for stage and differentiation grade (17 vs. 31 months, hazard ratio: 0.478, p=0.024). In both groups, aCGH revealed comparable molecular cytogenetic patterns of gain and losses. Only four significant differences were discriminated on chromosome regions 9q21.13, 9q22.1, 9q33.1 (all loss) and 19p13.2 (gain). Hierarchical clustering and principal component analysis did not relate to the clinically relevant subgroups.

Conclusion

Distinction between pancreatic adenocarcinoma and non-pancreatic periampullary cancers using aCGH analysis is not clinically useful. Although these tumors have a different biologic behaviour, they appear to be very similar at the molecular cytogenetic level.

Introduction

Pancreatic cancer is a major cause of cancer related death and its incidence is expected to increase in the next decade[1]. The majority of these tumors arise in the pancreatic head near the ampulla of Vater and present mainly by obstructive jaundice. In most cases these tumors are of Pancreatic Ductal origin (PDAC). However, they can also originate from the distal common bile duct and ampulla of Vater, collectively described as Non-Pancreatic Periampullary Cancers (NPPC). Although histologically these tumors are very similar, they carry a different, more favourable, prognosis.

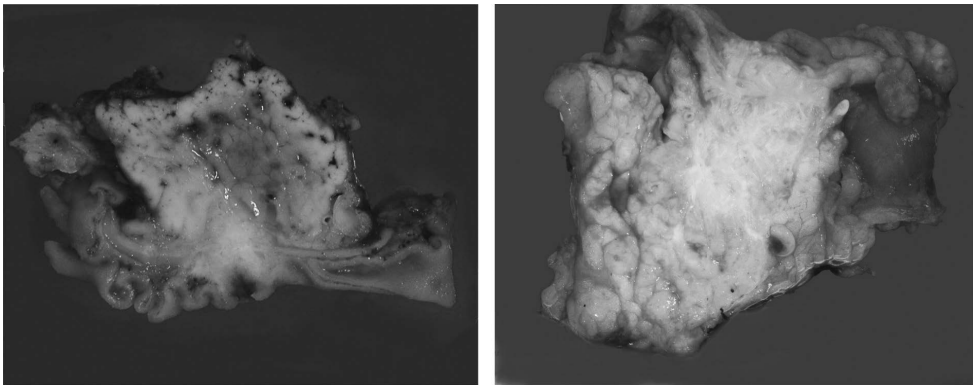
Different outcomes in trials exploring the value of adjuvant therapy after resection of PDAC or NPPC support the concept that these two diseases should be treated differently. Adjuvant chemotherapy has been shown to convey a survival benefit for PDAC in both CONKOO-1[2] and ESPAC-3[3] trials. There have been only a few randomized trials for adjuvant therapy in NPPC. In the largest trial for NPPC, the ESPAC-3 trial for Periampullary Cancer[4], the primary analysis failed to show a survival benefit of adjuvant chemotherapy. In another recent report on long-term follow-up of patients treated with or without adjuvant intra-arterial chemoradiotherapy it was demonstrated that patients suffering from NPPC might benefit from this type of adjuvant therapy in contrast to patients with PDAC[5].

The current pre-operative imaging of a tumor in the pancreatic head consists of multislice CT and endo-ultrasonography (EUS). These techniques are well capable to predict malignancy and resectability, but not of the exact origin of a tumor[6]. Most centers also perform routine EUS for pathological confirmation of cancer before commencing treatment. Pre-operative determination whether a pancreatic head mass is PDAC or NPPC is of great importance, in particular when neo-adjuvant regimens are considered. In addition, this may also be relevant in the palliative treatment of patients with suspected metastatic "pancreatic" cancer. Actually, it is not known exactly whether the patients that were included in trials for palliative chemotherapy, such as the FOLFIRINOX trial by Conroy et al.[7], suffered from metastastic PDAC or NPPC.

The aim of the current study was to explore molecular cytogenetic profiles, that could be used to distinguish between PDAC and NPPC.

Therefore we chose to use an array-based comparative genomic hybridization (aCGH) since this is a relatively simple, well known and validated technique. By using arrays spotted with nucleotide sequences of approximately 1000 kb, the full chromosomal DNA can be evaluated for imbalances, by comparing a sample with a standard human genome sample. These imbalances represent gains and losses of certain areas of the genome. The main advantages include filtering of background noise caused by normal cells in the sample, relatively simple procedure and wide availability[8]. Furthermore, Kitoh et al. showed that material obtained by fine needle biopsy at EUS can be successfully used for Comparative Genomic Hybridization (CGH) analysis [9]. This was a secondary prerequisite for possible pre-operative or pre-treatment differentiation.

Figure 1: NPPC (left), note the papilla of Vater at the duodenal/bottom side of the specimen, the tumor is located at the center of the ampulla. PDAC (right) A more diffuse tumor can be seen in the center of the pancreatic head. There is no relation with the ampulla or bile duct. It is also clear that multiple slicing is essential to evaluate the relation of a tumor to ductal structures.



Materials and Methods

Patients

From our archives we selected 50 samples of both PDAC (25) and NPPC (25) (distal CBD, Ampulla). All cases were reviewed macro- and microscopically by an experienced pancreato-biliary pathologist (HvD). And those cases were selected where there was no doubt whether the origin of the tumor was in or outside the pancreas. Therefore large tumors with distorted anatomy were excluded. A resection was considered radical when no tumor was present < 1 mm from the stained resection margin.

Array-Based CGH.

DNA was extracted from the paraffin-embedded, formalin-fixed tissue blocks using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). For this purpose, a hollow bore (1 mm in diameter) attached to a microscope was used to punch out cores of cancer tissue from selected tumor regions containing more than 80% tumor cells. The array-based CGH procedure and analysis was performed as previously described[8]. Slides containing triplicates of approximately 3,500 large insert clones spaced at density over the full genome were produced in the Leiden University Medical Center (Leiden, The Netherlands). The particular clone set used to produce these arrays is distributed to academic institutions by the Welcome Trust Sanger Institute (http://www.ensembl.org) at no cost and contains targets spaced at a density of about 1 Mb over the full genome, a set of subtelomeric sequences for each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis. DNA amplification, spotting on the slides, and hybridization procedures were based on protocols previously described[10]. We used commercially available male and female genomic DNA (Promega, Leiden, The Netherlands). Tumor and reference DNA were labeled with Cy3- and Cy5-dCTPs (Amersham Bioscience, Roosendaal, The Netherlands), respectively. After hybridization, the slides were scanned with a ScanArray Express HT (Perkin Elmer Life Sciences, Boston, MA, USA) to collect 16-bit TIF images through Cy3 and Cy5 filter sets. The spot intensities were measured with GenePixPro 5.0 software (Axon Instruments, Leusden, TheNetherlands). Spots outside the

20% confidence interval of the average of the replicates were excluded from the analyses. Gender mismatch with male or female reference DNA was used as an internal CGH control. Only samples showing a clear gender mismatch were included in the evaluation.

Statistical analyses.

We performed a straightforward clone-by-clone comparison to determine clones that were differently altered between the two groups. First, we calculated the $\log^2$ ratios of chromosomal gains and losses by an algorithm using flexible thresholds based on the standard deviation of the data sets of the specimens. Clones with aberration frequencies above or below 15% were listed as gains and losses, respectively. Statistical analyses were performed using SPSS statistical software, version 20 (IBM-SPSS Inc., Chicago, IL, USA). In addition we used dedicated analysis software to further explore the aCGH data. Partek (Partek Inc. Missouri, USA) for the Primary Components Analysis (PCA) and OmniViz (Omniviz Inc., MI, USA for clustering analysis). Online databases (www.ensembl.org; www.genecards.org) were used to correlate relevant regions to cancer associated genes. The Chi-square test was used for categorical and student-t for continuous variables. A p-value <0.05 (two-sided) was considered statistically significant. All p-values were rounded to three decimals. Survival was estimated using the Kaplan Meier method, significance calculated by log-rank test. In addition, near-significant factors ($p< 0.100$) from the univariate analysis were entered in a multivariate Cox-proportional hazards model, in accordance with the criteria for Proportional Hazards[11].

Results

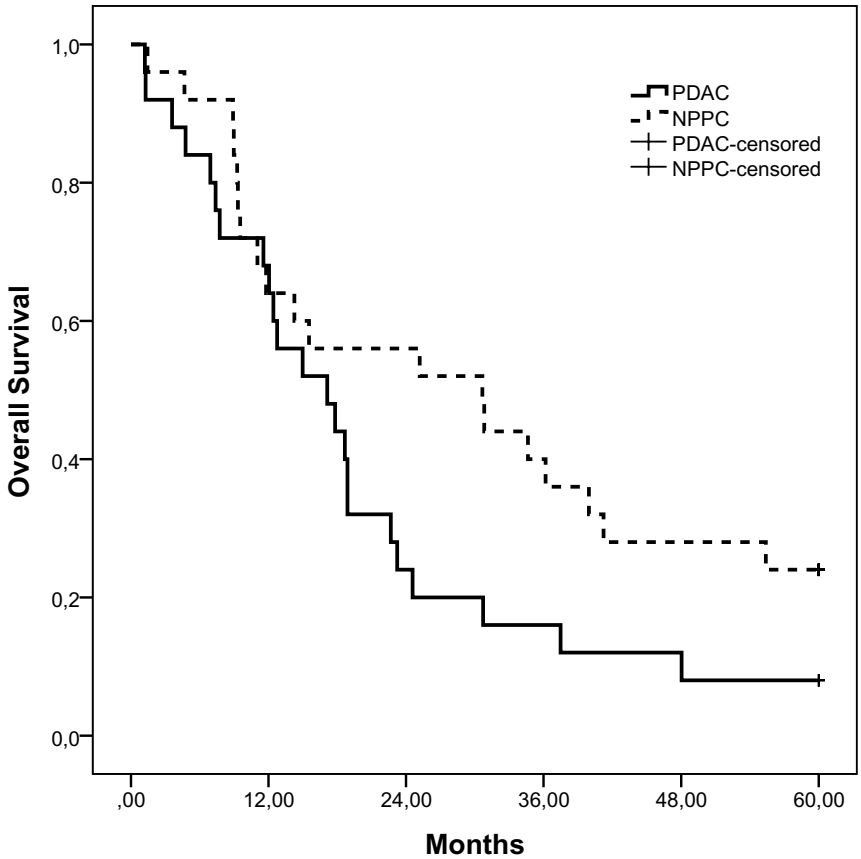
Clinicopathological data and survival.

The PDAC and NPPC group did not statistically significantly differ with regard to nodal status, percentage of radical resection or differentiation grade (Table 1). Patients suffering from NPPC had a longer median survival than patients with PDAC (Figure 2). In either group, one patient underwent an irradical resection (R1) at the margin of the superior mesenteric vein. All other R1 resections were caused by positive circumferential margins. Adjuvant chemotherapy was administered to 18 patients, as shown in Table 1. Most patients received intra-arterial chemotherapy, three patients received other experimental regimens. Chemotherapy did not influence survival. Most patients died of cancer-related causes, four patients died of other causes: One in the NPCC and three in the PDAC. Causes of death included pneumonia (n=1) , ischemic heart disease n=2 and severe pancreatitis of the pancreatic remnant n=1. Recurrences included liver metastases with or without local recurrence in most cases After entering the near significant ($p < 0.100$) univariate variables in to a Cox-regression model. It became clear that nodal status, differentiation grade (well-moderate and poor) and tumor origin (NPPC or PDAC) were independent and statistically significant variables (Table 2). T stage is differentially defined for PDAC and NPPC therefore it could not be included or tested in this model.

Table 1: Patient baseline characteristics T: Tumor stage, N: Nodal stage, G: grade, M: distant metastasis, R: radical resection, Adjuvant: adjuvant therapy, nt: not tested

Patient Baseline Characteristics			
	PDAC	NPPC	p
Male/ Female	10/15	9/16	1.000
Age	63	67	0.169
Adjuvant	8	5	0.520
T2		5	nt
T3	22	13	
T4	3	7	
N(+)	14	14	1.000
M	0	0	
G1	1	2	0,693
G2	17	18	
G3	6	5	
R0	23	21	0,667
R1	2	4	

Table 2: Survival for NPPC is better than for PDAC, (log-rank p=0.066, cox regression p=0.024)



Patients at risk						
Months	0	12	24	36	48	60
NPPC	25	16	14	10	7	6
PDAC	25	17	6	4	3	2

Table 2: Univariate and multivariate survival analysis for PDAC and NPPC
T: Tumor stage, N: Nodal stage, G: grade, M: distant metastasis, R: radical resection, Adjuvant: adjuvant therapy, nt: not tested

Survival analysis					
	Kaplan-Meier, months (95%)			Cox-regression	
	PDAC	NPPC	p-value	B(exp)	p-value
Male/ Female			0.259		
Age					
Adjuvant			0.843		
Pathology					
T2			nt		
T3					
T4					
N(+)	14 (7-21)	23 (6-41)	0.059	2.255	0.014
M					
G1	9 (6-12)	23 (14-31)	0.037	2.641	0.010
G2					
G3					
R0			0.866		
R1					
PDAC vs. NPPC	17 (9-25)	31 (6-56)	0.066	0.478	0.024

Results from the array Comparative Genomic Hybridization

aCGH revealed that both PDAC and NPPC share many common genomic imbalances (Figure 3, Table 3). Gains were detected on chromosome arms 1q, 2q, 6p, 7q, 8q, 11q, 12q, 17q, 18p, 19p, 19q and 20q, whereas losses were disclosed on chromosome arms 3p, 4q, 8p, 9p, 9q, 17p and 18q. Aberration frequencies above or below 15% are shown Table 3. Further, clone by clone analysis revealed four significant differences for loci on 9q21.13, 9q22.1, 9q33.1 (all losses) and 19p13.2 (gain) as shown in Table 4 (the relevant areas are also indicated with an arrow in Figure 3). Analysis of these clones did not reveal the presence of any known cancer-associated genes. Only a pseudogene was situated near clone RP11-563H8/ locus 9q21.13. Further exploration of the data by hierarchical clustering could not identify any (significant) subgroups of PDAC or NPPC. Analysis by Primary Component Analysis (PCA) did not reveal any major components or directions of grouping and therefore did not indicate significant subgroups. Figure 4 shows the results of the PCA analysis, each axis represents one of the first three principal components (X: 14.6%,Y: 9.23% ,Z: 7.03% of variance). No principal component could separate the main groups NPPC (blue) or PDAC (red), as can be seen by the scattered distribution. This implies that despite the presence of many genomic alterations at a molecular cytogenetic level no genes were disclosed for use in a clinical setting.

Table 3: PDAC and NPPC, frequent gains and losses

PDAC		NPPC	
Loss	Gain	Loss	Gain
			1q21.3-q22
			1q32.1
			1q41-q42.13
	2q36.3-q37.1		2q36.3-q37.1
		3p24.1	
		3p24.3	
4q22.1		4q22.1-q22.2	
		4q34.3-q35.1	
	6p21.31-p21.32		6p21.31-p21.32
	7q22.1		7q22.1
	7q36.1		7q36.1
8p22-p23.2		8p22-p23.2	
			8q24.13-q24.21
9p21.1-p24.3		9p13.3-p24.3	
9p11.2-p13.1		9p11.2-p13.1	
		9q12-q22.1	
		9q32-q33.1	
	11q12.1-q13.3		11q12.2-q13.2
	12q13.3		12q13.2-q13.3
		17p12-p13.1	
			17q11.1-q11.2
	17q12-q21.33		17q12-q21.33
			17q25.1-q25.2
	18p11.22		
18q11.2-q21.33		18q11.2-q23	
18q22.2-q23			
	19p13.11-p13.3		19p13.11-p13.2
	19q13.11-q13.32		19q13.11-q13.33
	20q11.1-q11.23		20q11.1-q11.22
	20q13.12-q13.13		20q13.12-q13.2

Table 4: Significant differences in gains and losses by clone, PDAC and **NPPC.**

Area	Clone	PDAC(%)	NPPC(%)		p-value
19p13.2	CTD-2231E14	0(0)	9(36)	Gain	0.002
9q21.13	RP11-563H8	1(4)	11(44)	Loss	0.002
9q22.1	RP11-176L21	0(0)	7(28)	Loss	0.01
9q33.1	RP11-451E16	4(16)	14(56)	Loss	0.007

Figure 3: Comparable frequency-plots for PDAC (upper) and NPPC (lower). Percentage gains (dark grey) and losses (light grey) per chromosome (x-axis, 1-22XY). Arrows indicate the areas of significant differences on chromosomes 9 and 19, i.e. loci on chromosome arms 9q21.13, 9q22.1, 9q33.1 (loss) and 19p13.2 (gain).

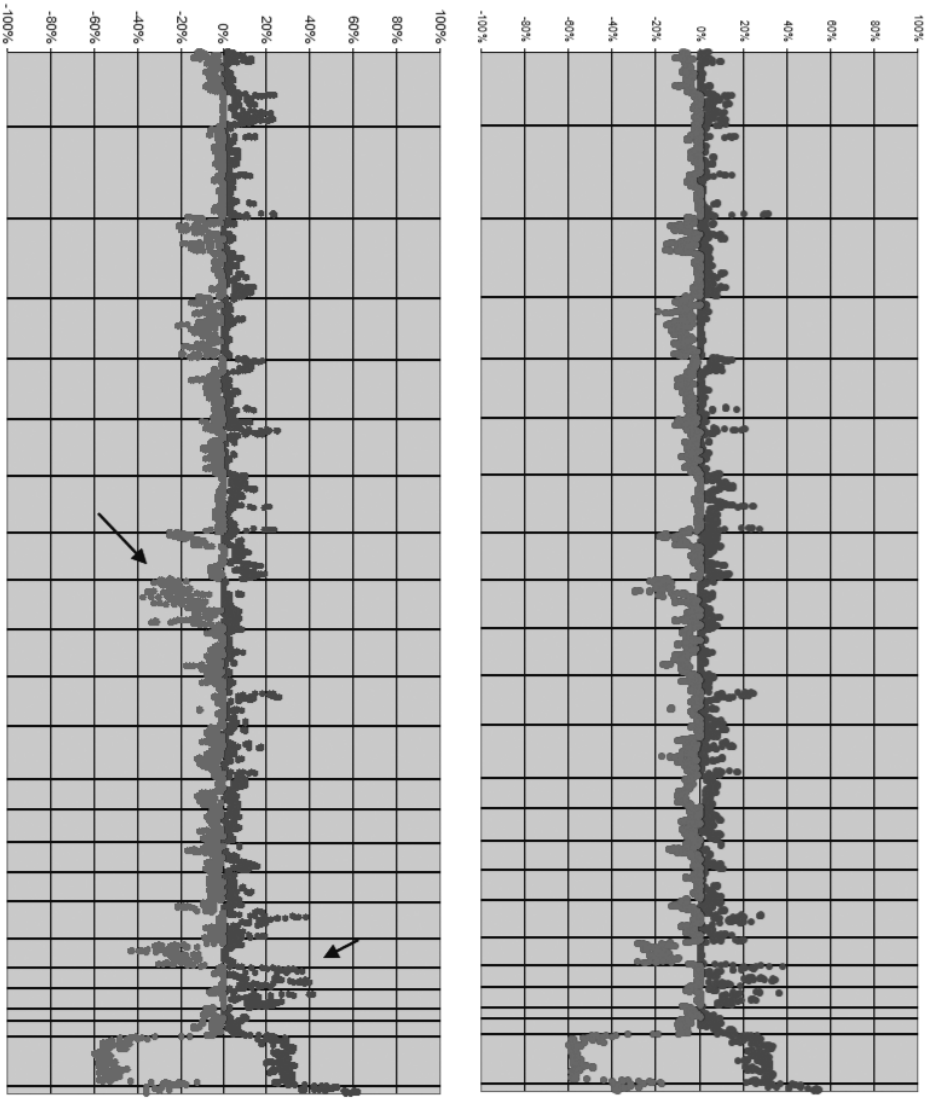
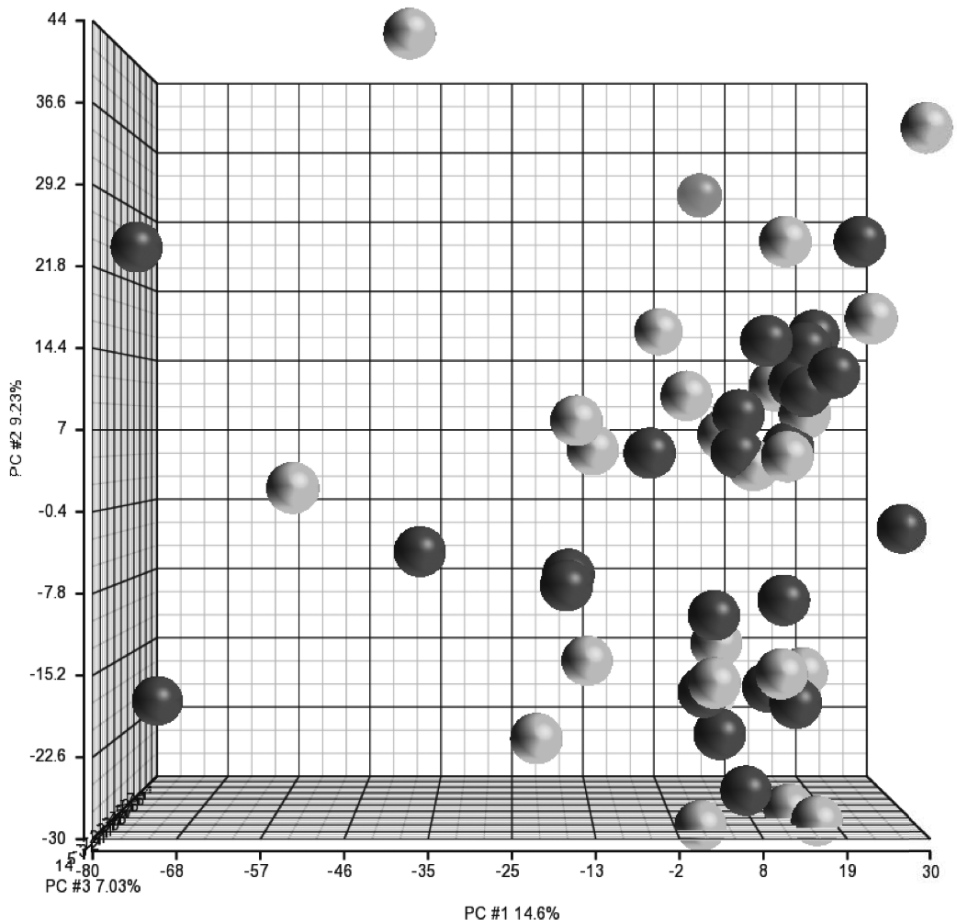


Figure 4: Principal Component Analysis (PCA), each axis represents one of the first three pricipal components (X: 14.6%,Y: 9.23% ,Z: 7.03% of variance). No principal component could separate the main groups NPPC (light grey) or PDAC (dark grey), as can be seen by the scattered distribution.



Discussion

Patients suffering from NPPC have a better prognosis than PDAC. Furthermore, response to adjuvant chemotherapy is different between these groups as was shown in the aforementioned randomized trials. Therefore these two groups represent separate families of tumors with distinct biological behaviour, and require a different adjuvant approach.

The clinical data as presented in the current study further underline this hypothesis. Our multivariate analysis confirms that the origin of the tumor is an independent significant indicator of prognosis. The effect of nodal status and differentiation grade - both markers of aggressive tumor biology - must also be taken into account. This observation is supported by other reports in the literature[12]. The independent effect of tumor origin in this model with other factors such as nodal status and differentiation grade in addition to similar tumor stages in the baseline analysis clearly indicates that it is the origin itself that influences survival and not early discovery due to the anatomical location of these tumors.

In addition to these clinical observations there are previous studies that have shown that there are molecular differences between PDAC an NPPC. For instance in the studies by Smeenk and Friess et al. where it was revealed that there is differential expression of the Erb-B family of growth factor receptors[13,14].

Previous reports of CGH studies comparing PDAC and NPPC are sparse. Most reports cover analysis of in-vitro cell lines, actual tumor specimens are rarely used for CGH analysis, and if so, they lack correlation with clinical parameters. In PDAC and NPPC many common losses are found on chromosomes 1p, 1q, 3p, 4p, 4q, 5p, 5q, 6p, 6q, 7p, 7q, 8p, 8q, 9p, 9q, 11p, 11q, 13q, 14q, 15q, 16p, 17p, 17q, 18q, 19p, 19q, 21p, 21q, 22p and 22q. Common gains are found on 1p, 1q, 2p, 2q, 3q, 5p, 5q, 7p, 7q, 8p, 8q, 10q11p,12p13q, 14q, 15q, 18q, 20p and 20q9,15,16. Only one small study directly compared both tumor types by CGH[17]. Besides expected genome wide genetic alterations, only a few significant differences were found:

gains on 1q25, 7p15, 8q23 and 3p21, and losses on 17q24 occurred more frequently in NPPC. Unfortunately the correlation with clinical parameters or survival was not investigated. The apparent differences between the aforementioned and the present study may be caused by differences in methods such as laser capture vs. hollow-bore, ethnicity of the studied patients and odds, since the study groups are relatively small and the number of analyzed clones is very large. In the present study we also observed some significant differences between PDAC and NPPC. Although theoretically these loci may harbour genes that could be implicated in the tumorigenesis and or biological behaviour of these tumours, the regions we found (9q21.13, 9q22.1 , 9q33.1 and 19p13.2) do not carry known onco-(suppessor) genes.

It was surprising to find that NPPC and PDAC groups are very similar at the level of an aCGH analysis. It can be argued that the sample size of this study was to small. However our main interest was to find specific gains and losses to differentiate the relevant clinical subgroups PDAC an NPPC pre-operatively in biopsies taken by EUS. The significant genetic differences we found are relatively infrequent (28-56%), and therefore lack the specificity and sensitivity to draw pre-operative conclusions wich may guide therapy. By an increase in sample size it may be possible to identify other subgroups or significant imballances, although it is unlikely that these would have the specificity or sensitivity for routine clinical use.

We conclude that on a molecular cytogenetic level of aCGH analysis, PDAC an NPPC are actually very similar. This may, in part, be due to our DNA-based array exploration itself. Further studies should therefore include expression arrays to detect more subtle RNA-based genetic differences.

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Chapter 7

Type I Interferons in the Treatment of Pancreatic Cancer: Mechanisms of Action and Role of Related Receptors

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Abstract

Objective: We evaluated the role of type I interferons (IFNs) and IFN receptors in the regulation of cell growth in 3 human pancreaticadenocarcinoma cell lines (BxPC-3, MiaPaCa-2, and Panc-1).

Background: Chemotherapy and radiotherapy have a marginal role in the management of pancreatic adenocarcinoma. The addition of IFN-α showed promising results in early clinical trials.

Methods: Cell proliferation and apoptosis were evaluated by DNA measurement and DNA fragmentation, respectively. Type I IFN receptor (IFNAR-1 and IFNAR-2 subunits) was determined by quantitative RT-PCR and immunocytochemistry. Cell cycle distribution was evaluated by propidium iodide staining and flowcytometric analysis.

Results: The incubation with IFN-β for 6 days showed a potent inhibitory effect on the proliferation of BxPC-3 (IC50, 14 IU/mL) and MiaPaCa-2 (IC50, 64 IU/mL). The inhibitory effect of IFN-β was stronger than IFN-α in all 3 cell lines and mainly modulated by the stimulation of apoptosis, although cell cycle arrest was induced as well. The expression of the type I IFN receptors was significantly higher in BxPC-3 (the most sensitive cell line to IFN) and mainly localized on the membrane, whereas in Panc-1 (the most resistant cell line) about 60% to 70% of cells were negative for IFNAR-2c with a mainly cytoplasmic staining for IFNAR-2c.

Conclusion: The antitumor activity of IFN-β is more potent than IFN-α in pancreatic cancer cell lines through the induction of apoptosis. Further studies should investigate in vivo whether the intensity and distribution of IFNAR-1 and IFNAR-2c may predict the response to therapy with IFN-α and IFN-β in pancreatic cancer.

Introduction

Pancreatic adenocarcinoma is a highly aggressive malignancy[1]. Surgery is the only curative therapy. Unfortunately, only 5% to 15% of patients are surgical candidates at the time of the diagnosis due to a lack of specific symptoms, limitations in diagnostic methods, and the biologically aggressive nature of this tumor.1 In this selected group of patients, adjuvant chemotherapy has a survival benefit but the 5-year survival of 21%, as described by the European Study Group for Pancreatic Cancer, remains poor[2]. The role of chemoradiotherapy in the management of pancreatic adenocarcinoma is unclear[3]. However, it has been recently described[4] that interferon (IFN)-α in combination with adjuvant chemoradiotherapy improved 5-year survival to 55%.

In vitro and in vivo studies have demonstrated the efficacy of type I IFNs (eg, IFN-α, IFN-β, IFN-ω, IFN-κ, and IFN-τ), in the treatment of several tumors[5-9].5 Although the antitumor effects of IFN-α have been studied in detail, those of IFN-β are not well clarified. IFN-β is a multifunctional cytokine that binds the same receptor of IFN-α, but with higher affinity[10]. It seems to be an essential mediator not only for the innate immune responses against microbial infections, but also for a host defense system against oncogenesis[6,11]. Moreover, several studies showed that IFN-β has greater antitumor effects than IFN-α[10,12-16]. On the basis of these observations, IFN-β represents a promising drug in the treatment of cancer.

Importantly, several chromosomal aberrations have been detected in pancreatic adenocarcinoma, including a frequent loss of chromosome arm 9p, observed in more than 80% of human pancreatic cancer[17]. Together with the tumor-suppressor genes p16INK4a, p15INK4b, and p14ARF also the IFN-α and IFN-β genes are located on chromosome 9p[18]. Therefore, in relation to the defensive role of IFNs against tumors[11], the absence of the expression of IFNs may have an important role in the pathogenesis and probably in the treatment of pancreatic adenocarcinoma.

To further explore the possibilities of new medical treatments in pancreatic cancer, we evaluated in the present study the antitumor activity of IFN-α and IFN-β in 3 human pancreatic adenocarcinoma cell lines (BxPC-3, MiaPaCa-2, and Panc-1), as well as the role of IFN receptors in the responsiveness to type I IFNs.

Methods

Cell Lines and Culture Conditions

The human pancreatic cell lines, BxPC-3, MiaPaCa-2, and Panc-1 were purchased from the American Type Culture Collection. The cells were cultured in a humidified incubator containing 5% CO2 at 37°C. The culture medium consisted of RPMI 1640 supplemented with 10% FCS, penicillin (1×10^5 U/L) and l-glutamine (2 mmol/l). Periodically, the cells were confirmed as Mycoplasma-free. Cells were harvested with trypsin (0.05%), EDTA (0.02%), and resuspended in medium. Before plating, the cells were counted microscopically using a standard hemocytometer. Trypan Blue staining was used to assess cell viability and always exceeded 95%. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

Drugs and Reagents

Human recombinant IFN-α-2b (Intron-A) was obtained from Schering-Plough Corporation (Utrecht, The Netherlands), while human recombinant IFN-β-1a was acquired from Serono Inc. (Rebif, Rockland, MA). All compounds were stored at −20°C, and the stock solution was constituted in distilled water according to the manufacturer instructions.

Cell Proliferation Assay

After trypsinization the cells were plated in 1 mL of medium in 48-well plates at a density of 5×10^3 to 4×10^4 cells/well, depending on the length of the incubation period. The plates were then placed in a 37°C, 5% CO2 incubator overnight. The next day the cell culture medium was replaced with 1 mL/well medium containing

confluence. For 6-day experiments, the medium was refreshed after 3 days and compounds were added again. Measurement of total DNA contents, representative for the number of cells, was performed using the bisbenzimide fluorescent dye (Hoechst 33258) (Boehring Diagnostics, La Jolla, CA), as previously described.[19]

Measurement of DNA Fragmentation (Apoptosis)

Cells (10^4 to 4×10^4)/well, depending on the length ofthe incubation period, were plated on a 48-well plate andthe cells were allowed to adhere overnight. The next day, the cell culture medium was replaced with 1 mL/well medium containing increasing concentrations (0–10,000 IU/mL) of IFN-α or IFN-β. Each treatment was performed in quadruplicate. After an additional incubation of 1 and 3 days, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISAPlus, Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used, as previously described.[20] Relative apoptosis was determined by calculating the ratio of the average absorbance of the treatment wells to the average absorbance of the control wells. The data were corrected for the effect on cell number after 1 and 3 days of treatment.

Cell Cycle Analysis

Cells (1 to 4×10^6) depending on the length of the incubation period, were plated in 75-cm2 culture flasks (Corning Costar, Amsterdam, The Netherlands). After 1 day medium was changed with fresh medium (control group) and with fresh medium plus IFN-α or IFN-β at the concentration of 1000 IU/mL. Each treatment was performed in duplicate. After 1, 2, and 3 days of incubation, the cells were harvested by gentle trypsinization and prepared for cell cycle determination using propidium iodide for DNA staining, as previously described.[16] The stained cells were analyzed by FACS-calibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) and CellQuest Pro Software. Cell cycle progression was measured with corresponding absorbances for G0/G1, S and G2-M phases, whereas apoptosis was measured by quantifying the sub-G0 peak.

Quantitative RT-PCR

The expression of type I IFN receptors (IFNAR-1, IFNAR-2 total, the short form IFNAR-2b, and the long form IFNAR-2c) and housekeeping gene hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA was evaluated by quantitative RT-PCR in all 3 pancreatic cancer cell lines, as previously described.[16] Briefly, poly A+ mRNA was isolated using Dynabeads Oligo (dT)25 (Dynal AS, Oslo, Norway) from cell pellets containing approximately 5×10^5 cells. Complementary DNA (cDNA) was synthesized using the poly A+ mRNA in a Tris-buffer together with 1 mmol/L of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 40 µL. This mixture was incubated for 1 hour at 42°C. One fifth of the cDNA library was used for quantification of IFN receptors and HPRT mRNA levels.

A quantitative PCR was performed by AmpliTaq Gold DNA Polymerase and the ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Groningen, The Netherlands) for real-time amplifications, according to the manufacturer's protocol. Each sample was assayed in duplicate. The assay was performed using 15 µL TaqMan Universal PCR Master Mix (Applied Biosystems, Capelle aan de Ijssel, The Netherlands), forward primer, reverse primer, probe, and 10 µL cDNA template, in a total reaction volume of 25 µL. PCR amplification started with a first step for 2 minutes at 50°C, followed by an initial heating at 95°C for 10 minutes, samples were subjected to 40 cycles of denaturation at 95°C for 15 seconds and annealing for 1 minute at 60°C.

The primer and probe sequences that were used for the detection of IFNAR-1, IFNAR-2 total, IFNAR-2b, IFNAR-2c, and HPRT have been previously described.[16] All the primer and probe sequences were purchased from Biosource (Nivelles, Belgium).

The detection of HPRT mRNA was used for normalization of IFN receptor mRNA levels. Expression of IFNAR-2a mRNA, the soluble form of IFNAR-2 subunit, was determined indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total.

To exclude contamination of the PCR reaction mixtures, the reactions were performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of HPRT and type I IFN receptors human cDNA was amplified in parallel with the cDNA samples.

Immunocytochemistry

Cytospin preparations of BxPC-3, MiaPaCa-2, and Panc-1 cells were fixed with acetone for 10 minutes. After washing 2 times with PBS, the cells were incubated for 30 minutes at room temperature with antibodies to human IFNAR-1 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and IFNAR-2c (monoclonal antibody, Dr E. Croze, Berlex Biosciences, Richmond, CA) subunits, and for overnight with antibodies to IFNAR-2b (rabbit polyclonal antibody, Santa Cruz Biotechnology, Inc.). Finally, a peroxidase complex for IFNAR-1 and IFNAR-2b, or standard streptavidin-biotinylated alkaline phosphatase (both from IL Immunologic, Duiven, The Netherlands) for IFNAR-2c, were used according to the manufacturer's recommendations to visualize the bound antibodies.

Negative controls for the immunohistochemistry included: 1) omission of the primary antibody; and 2) preabsorption of the antibody for IFNAR-2b with the respective immunizing receptor peptide.

Statistical Analyses

All experiments were carried out at least 3 times and gave comparable results. For statistical analysis GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) was used. Fifty percent growth-inhibitory concentrations (IC50) and maximal inhibitory effects were calculated using nonlinear regression curve-fitting program. The comparative statistical evaluation among groups was first performed by the ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The unpaired Student t test was used to analyze the differences in concentration-effect curves (IC50 and maximal inhibitory effect) and effects in cell cycle modulation between different

types of IFNs, and the differences of the growth inhibitory effects of IFNs after 3 and 6 days of treatment. Correlation analyses were performed using Pearson's coefficients.

In all analyses, values of $P < 0.05$ were considered statistically significant. Data are reported as mean ± SEM. Statistical analysis was made after logarithmic transformation.

Results

Antiproliferative Effects of Type I IFNs

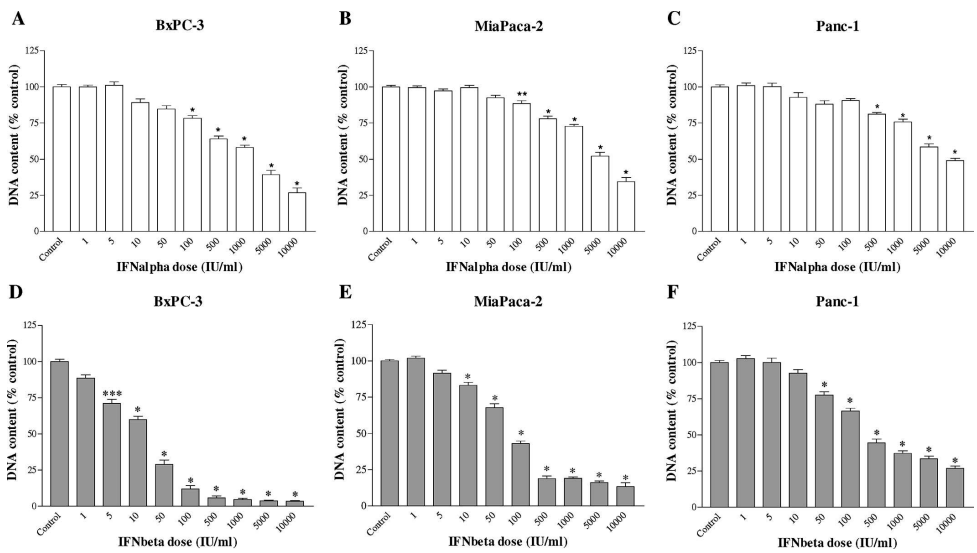
After 6 days of incubation, IFN-α and IFN-β significantly suppressed the growth of all 3 pancreatic cancer cell lines in a dose-dependent manner (Fig. 1), with a mean IC50 of 606 IU/mL and 14 IU/mL in BxPC-3, respectively; 1531 IU/mL and 64 IU/mL in MiaPaCa-2, respectively; and 1250 IU/mL and 112 IU/mL in Panc-1, respectively.

The growth-inhibitory effect of IFN-β was significantly more potent than that of IFN-α, as shown by the higher maximal inhibition of proliferation induced by IFN-β compared with IFN-α (96.7% ± 2% and 72% ± 5.7%, respectively, $P < 0.0001$ in BxPC-3; 87.5% ± 3.2% and 69.1% ± 6.1%, respectively, $P < 0.0001$ in MiaPaCa-2; 70.7% ± 1.4% and 53% ± 5.7%, respectively, $P < 0.0001$ in Panc-1) after 6 days of treatment, as well as by the lower logIC50 of IFN-β compared with IFN-α (1.15 ± 0.06 and 2.78 ± 0.15, respectively, $P < 0.00001$ in BxPC-3; 1.8 ± 0.07 and 3.18 ± 0.13, respectively, $P < 0.00001$ in MiaPaCa-2; 2.05 ± 0.05 and 3.1 ± 0.17, respectively, $P < 0.0001$ in Panc-1). In BxPC-3 and MiaPaCa-2, IFN-β induced a statistically significant cell growth inhibition already at very low concentrations (5–10 IU/mL).

In all 3 pancreatic cell lines, the effects of IFN-α and IFN-β were time-dependent. Indeed, the maximal inhibition of cell proliferation, induced by both cytokines, was higher after 6 days compared with 3 days of incubation (both $P < 0.0001$ in BxPC-3; $P < 0.005$ and $P < 0.0001$, respectively, for IFN-α and IFN-β in MiaPaCa-2; both $P < 0.0001$ in Panc-1). In addition, there was no difference in IC50 values after 3 and 6 days of incubation with IFN-α or IFN-β in the 3 cell lines (data not shown).

The cell lines exhibited different sensitivities to the treatment, particularly with IFN-β. BxPC-3 resulted to be the most sensitive and Panc-1 the most resistant. The maximal inhibition of proliferation for IFN-β was higher in BxPC-3 compared with MiaPaCa-2 ($P < 0.05$) and Panc-1 ($P < 0.001$), while it was lower in Panc-1 compared with MiaPaCa-2 ($P < 0.01$). Similarly, the IC50 of IFN-β was significantly lower in BxPC-3 than in MiaPaCa-2 and Panc-1 (both $P < 0.001$), and higher in Panc-1 compared with MiaPaCa-2 ($P < 0.05$). The maximal inhibition of proliferation for IFN-α was higher in BxPC-3 compared with Panc-1 ($P < 0.05$), while no difference in IC50 values of IFN-α was observed between the 3 cell lines.

Figure 1: Effects of IFN-α (A–C) and IFN-β (D–F) treatment on cell proliferation, as measured by total DNA content, using Hoechst 33258. Pancreatic cancer cell lines were incubated for 6 days without (control) or with the drugs indicated at different concentrations. Values are expressed as the percentage of control (untreated cells) and represent the mean ± SEM of at least 3 independent experiments in quadruplicate. The mean DNA content in controls were: 2260 ng/well (IFN-α, BxPC-3), 2430 ng/well (IFN-β, BxPC-3), 8562 ng/well (IFN-α, MiaPaCa-2), 8803 ng/well (IFN-β, MiaPaCa-2), 4224 ng/well (IFN-α, Panc-1), and 4172 ng/well (IFN-β, Panc-1). *P < 0.001; **P < 0.01; ***P < 0.05 versus control.



Effects of Type I IFNs on Apoptosis

A crucial step in apoptosis is DNA fragmentation, a process that results from the activation of endonucleases, which degrade chromatin into smaller fragments. The measurement of DNA fragmentation was used to investigate the effect of treatment with IFN-α and IFN-β on apoptosis (Figs. 2 and 3).

After 1 day of incubation, IFN-α had no remarkable stimulatory effects on DNA fragmentation at any concentration up to 1000 IU/mL in all 3 cell lines, only at the very high dose of 10,000 IU/mL IFN-α induced a significant increase in DNA fragmentation (Fig. 2A–C). On the other hand, a dose-dependent induction of apoptosis was observed after IFN-β treatment in BxPC-3 and MiaPaCa-2, with a maximal increase of DNA fragmentation of about 3.5 times compared with the untreated control (Fig. 2D, E). This effect was already statistically significant at very low concentrations (5–10 IU/mL). In Panc-1, a stimulating effect on apoptosis was observed only for very high concentrations of IFN-β (≥500 IU/mL) (Fig. 2F).

After 3 days of treatment with IFN-α, an increase in DNA fragmentation was detected in BxPC-3 and MiaPaCa-2 at a moderate to high dose (Fig. 3A, B). Moreover, the induction of apoptosis by IFN-β remained high, with a maximal stimulation of about 4- and 10-fold, compared with the control, respectively, in MiaPaCa-2 and BxPC-3 (Fig. 3D, E). In addition, after 3 days of incubation, the stimulatory effects on apoptosis persisted in Panc-1 only at high doses of IFN-α (10,000 IU/mL, Fig. 3C) and IFN-β (≥500 IU/mL, Fig. 3F).

These data were also confirmed by morphologic observations. In all 3 cell lines, the treatment with IFN-β induced clear structural alterations consistent with apoptosis, such as cell shrinkage, pyknotic nucleus, and detachment from the plate after 1 to 3 days, also at very low doses in BxPC-3 cell line (not shown). These morphologic changes were evident only at high doses of IFN-α treatment.

The inhibitory effects of IFN-β on the cell growth of BxPC-3 and MiaPaCa-2 cell lines appeared to be mainly due to an early pro-apoptotic activity, as shown by

the highly significant positive correlation between cell proliferation inhibition after 6 days of treatment and DNA fragmentation induction after 1 day ($r^2 = 0.95$, $P < 0.0001$, both for BxPC-3 and MiaPaCa-2) and 3 days of incubation ($r^2 = 0.95$, $P < 0.0001$, for BxPC-3; $r^2 = 0.90$, $P < 0.0001$, for MiaPaCa-2). At this early time point, apoptosis seems to be not involved in the antiproliferative effect of IFN-α on pancreatic cancer cells. Indeed, no significant correlation has been observed between cell proliferation inhibition after 6 days and DNA fragmentation variation after 1 day of treatment with IFN-α. Only after 3 days of treatment with IFN-α, we observed a positive correlation between DNA fragmentation variation and the 6 days cell proliferation inhibition ($r^2 = 0.74$, $P < 0.0001$, for BxPC-3; $r^2 = 0.71$, $P < 0.0001$, for MiaPaCa-2).

Figure 2: Effects of IFN-α (A–C) and IFN-β (D–F) treatment on apoptosis (DNA fragmentation) in BxPC-3, MiaPaCa-2, and Panc-1 cell lines. The cells were incubated for 1 day without (control) or with the drugs indicated at different concentrations. Values are absorbance units and are expressed as percent of the control. Data are the mean ± SEM. *P < 0.001 versus control.

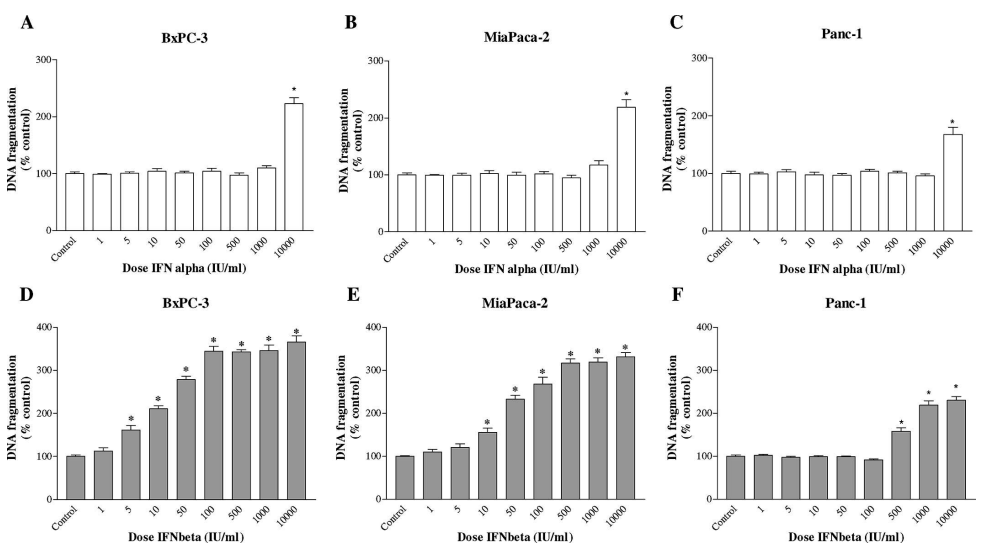
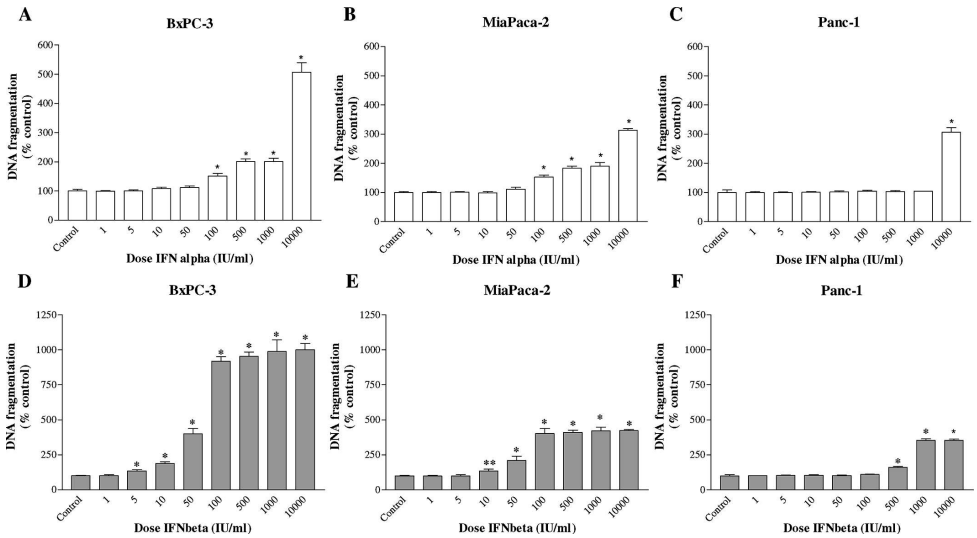


Figure 3: Effects of IFN-α (A–C) and IFN-β (D–F) on the apoptosis (DNA fragmentation) in BxPC-3, MiaPaCa-2, and Panc-1 cell lines. Pancreatic cancer cell lines were incubated for 3 days without (control) or with the drugs indicated at different concentrations. Values are absorbance units and are expressed as percent of the control. Data are the mean ± SEM. *P < 0.001; **P < 0.01.



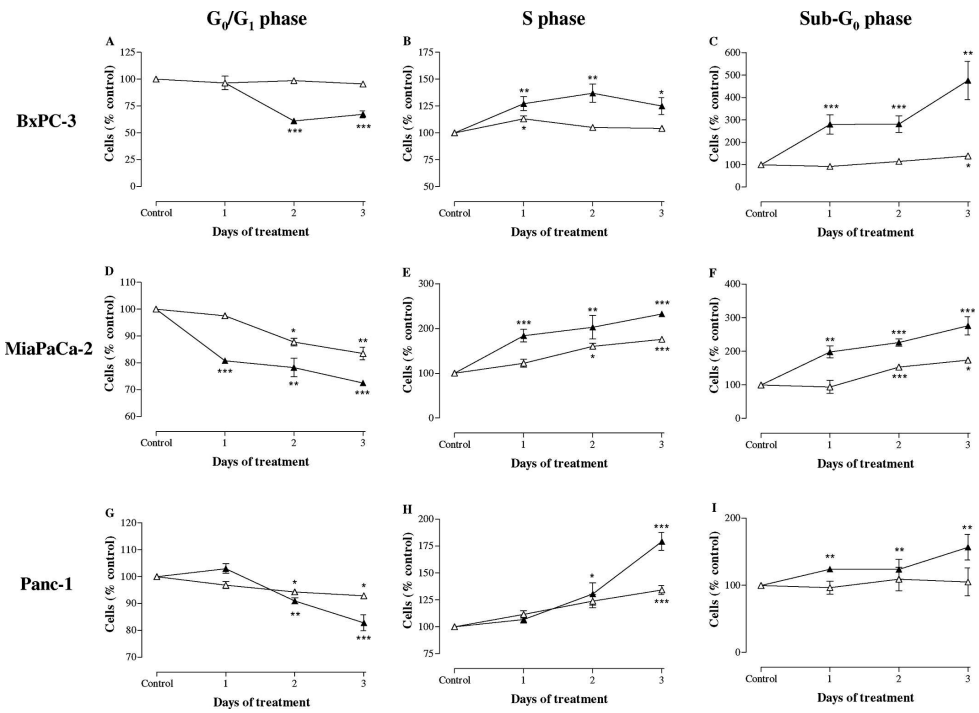
Effects of Type I IFNs on the Cell Cycle

We also evaluated the effect of treatment with IFN-α (1000 IU/mL) and IFN-β (1000 IU/mL) on cell cycle phase distribution after 1, 2, and 3 days of incubation in BxPC-3, MiaPaCa-2, and Panc-1 (Fig. 4A–I).

IFN-α treatment induced a significant accumulation in S phase compared with the control in all 3 cell lines and a decrease in the proportion of cells in G0/G1 phase in MiaPaCa-2 and Panc-1. In addition, the histograms of cell cycle revealed a late and slight increase in cells with subdiploid DNA content (sub-G0 phase) only in BxPC-3 and MiaPaCa-2, confirming the induction of apoptosis after IFN-α treatment, as previously shown by the analysis of DNA fragmentation. In a comparable manner, the incubation with IFN-β increased the fraction of all 3 cell lines in the S phase of the cell cycle, whereas the proportion of cells in G0/G1 phase decreased in comparison with the control. IFN-β induced a variable

accumulation of cells in sub-G0 phase in all cell lines (BxPC-3>MiaPaCa-2>Panc-1). These data suggest that pancreatic cancer cells in S phase fail to transit into G2 and M phases efficiently and exhibit a prolonged stay in S phase after treatment with type I IFNs. The cell cycle arrest induced by IFN-β was more potent than that of IFN-α, considering that the percentage of cells in S phase compared with the control was significantly higher after 3 days of incubation with IFN-β than after IFN-α (BxPC-3: $P < 0.05$, MiaPaCa-2: $P < 0.001$, Panc-1: $P < 0.001$).

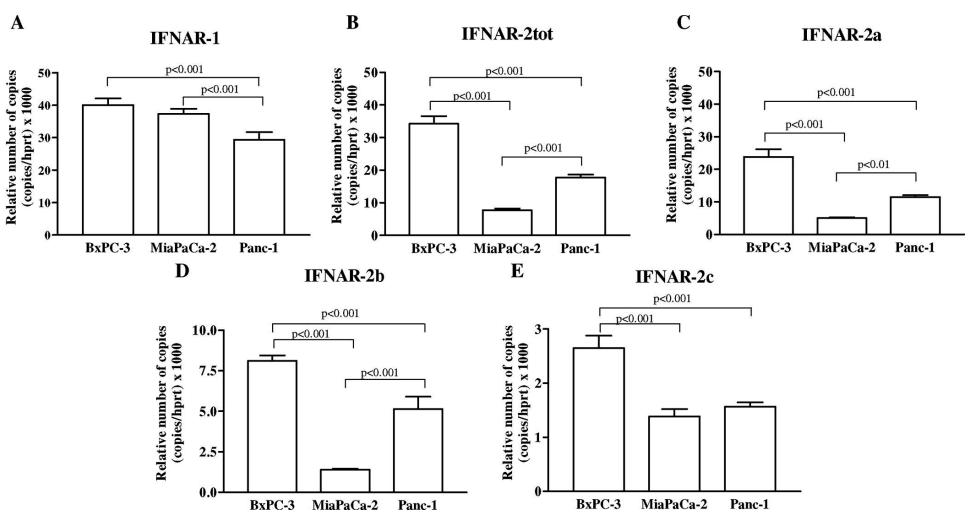
Figure 4: Cell cycle distribution after 1, 2, and 3 days of incubation with 1000 IU/mL IFN-α and 1000 IU/mL IFN-β in BxPC-3 (A–C), MiaPaCa-2 (D–F), and Panc-1 (G–I) cells. Data are expressed as mean ± SEM of the percentage of cells in the different phases of the cell cycle, as compared with untreated control cells. Control values have been set to 100%. Δ, IFN-α; ▲, IFN-β. *P < 0.05; **P < 0.01; ***P < 0.001 versus control.



Expression of Type I IFN Receptor mRNA

Since the susceptibility of cells to IFNs could reflect the different amount of corresponding receptors, we analyzed the expression of type I IFN receptors (IFNAR-1 and IFNAR-2, short and long form) mRNA by real-time quantitative RT-PCR in the BxPC-3, MiaPaCa-2, and Panc-1 cell lines. Using sequence specific primers against the type I IFN receptor subunits, we detected the presence of IFNAR-1, IFNAR-2 total, IFNAR-2b and IFNAR-2c mRNA, normalized for the amount of the housekeeping gene HPRT. As shown in Figure 5, the expression of IFNAR-1 mRNA was significantly higher in BxPC-3 and MiaPaCa-2 compared with Panc-1 (both $P < 0.001$), whereas no statistically significant difference was observed between BxPC-3 and MiaPaCa-2. In addition, the mRNA expression level of IFNAR-2a, IFNAR-2b, and IFNAR-2c mRNA was higher in BxPC-3 compared with MiaPaCa-2 and Panc-1 ($P < 0.001$).

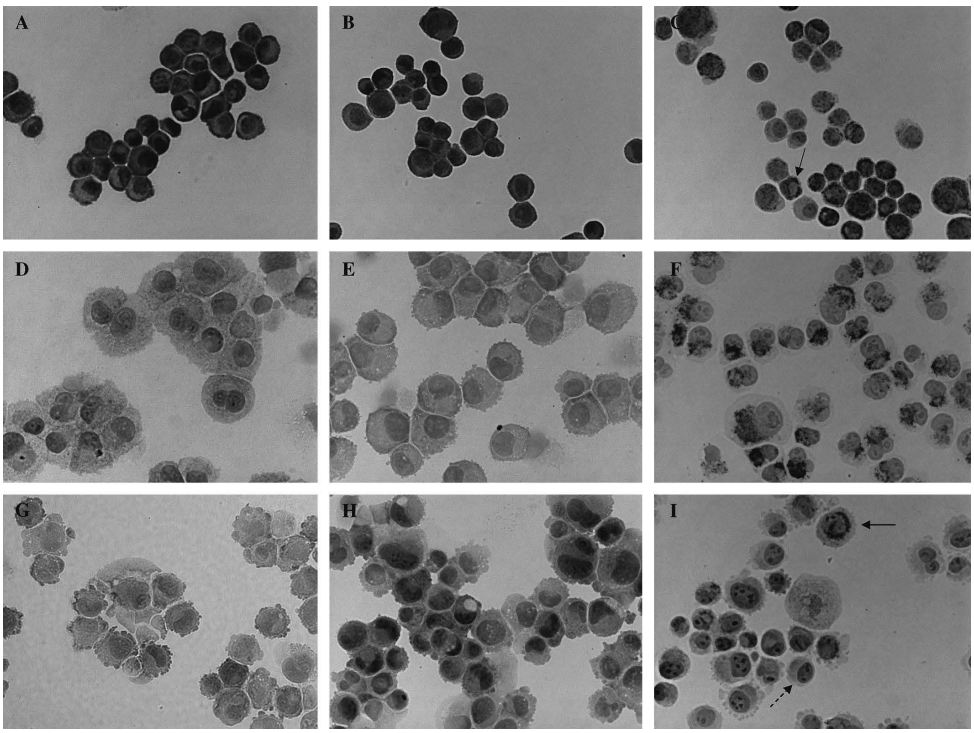
Figure 5: A–E, Relative expression level of type I IFN receptor (IFNAR-1, IFNAR-2 total, IFNAR-2a, IFNAR-2b, IFNAR-2c) mRNA normalized to HPRT mRNA in human pancreatic cancer cell lines (BxPC-3, MiaPaCa-2, Panc-1), evaluated by quantitative RT-PCR. Values represent the mean ± SEM.



Immunocytochemistry

Specific immunoreactivity for IFN receptor subunits (IFNAR-1, IFNAR-2b, and IFNAR-2c) was found in all 3 pancreatic cancer cell lines (Fig. 6A–I). It was strongly positive for IFNAR-1 in BxPC-3 (Fig. 6A) and the staining was predominantly at the plasma membrane. On the other hand, in MiaPaCa-2 (Fig. 6D) and in Panc-1 (Fig. 6G), the expression of IFNAR-1 was lower and particularly distributed in the cytoplasm, although a proportion of Panc-1 cells resulted to be mildly to moderately positive for IFNAR-1 at the plasma membrane as well. IFNAR-2b showed a comparable expression in BxPC-3 (Fig. 6B) and Panc-1 (Fig. 6H), while in MiaPaCa-2 (Fig. 6E) the expression of this subunit was lower compared with the other 2 cell lines. In BxPC-3 and in MiaPaCa-2, the immunostaining of IFNAR-2b was localized in the cytoplasm and on the membrane, while in Panc-1 the expression of IFNAR-2b was preferentially on the cytoplasm. IFNAR-2c is mainly expressed on the plasma membrane and in the cytoplasm in BxPC-3 (Fig. 6C) and in MiaPaCa-2 (Fig. 6F), respectively. Finally, in Panc-1 (Fig. 6I) this receptor subunit is primarily expressed in cytoplasm, the IFNAR-2c pattern is heterogeneous and the staining is negative in about 60% to 70% of the cells.

Figure 6: Immunocytochemical detection of IFNAR-1 (A, D, G), IFNAR-2b (B, E, H), and IFNAR-2c (C, F, I) receptors in BxPC-3 (A–C), MiaPaCa-2 (D–F), and Panc-1 (G–I). Original magnification, ×400. The expressions of IFNAR-1 and IFNAR-2c are mainly membranous in BxPC-3 (A and C, solid arrow). In Panc-1, IFNAR-2c is localized in the cytoplasm (I, solid arrow) and 60% to 70% of the cells are negative for IFNAR-2c (I, dashed arrow)



Discussion

Although few trials criticized the intense toxicity of IFNs,[21,22] Traverso's group showed that combination of IFN-α with adjuvant chemoradiation therapy may increase response rates and survival in patients with pancreatic cancer[4,23]. In addition, the administration of IFN-α in combination with 13-cis retinoic acid or with 5-fluorouracil, leucovorin, and cisplatin increased antitumor effect in advanced pancreatic carcinoma[24-26].

Whereas the role of IFN-α has been extensively studied, the effect of other type I IFNs on pancreatic cancer has been evaluated less extensively. Preliminary reports suggested the possibility to use IFN-β in the treatment of pancreatic cancer[27-30]. A high local production of IFN-β induced a strong antitumor effect on PANC02-H7 cells, a highly metastatic mouse pancreatic carcinoma cell line successfully transfected with a vector containing a murine IFN-β gene[27]. A recent paper showed that the treatment of human pancreatic cancer cell lines with gemcitabine and human IFN-β gene entrapped in liposomes was more effective than either treatment alone[28]. Busch et al described the stabilization of the disease in a patient with incomplete resection of a pancreatic cancer, treated with IFN-β in combination with gemcitabine, cisplatinum, and radiotherapy[29]. On the other hand, few long-lasting responses and disease stabilization have been achieved in patients with advanced pancreatic cancer by combining IFN-β with chemotherapy and retinoids[30]. However, the efficacy of IFN-β in the treatment of pancreatic cancer, the potential differences in antitumor activity with IFN-α, and the mechanisms of action that are involved are still poorly understood. Moreover, in clinical practice, one of the main limits of therapy with type I IFNs is the scanty availability of molecular predictors, potentially useful in deciding whether a patient should be treated. This is a crucial point, considering that several tumors are completely or partially resistant to IFNs. A recent in vitro study showed that IFNs have antiproliferative effects on pancreatic cancer cell lines expressing the IFNAR-2 subunit[31]. Besides, patients with pancreatic cancer who expressed IFNAR-2 represent about 25% of cases[32,33] and have better survival compared with patients who did not express this receptor.[33] However, IFNAR-2 receptor is not the only component modulating the antitumor activity of type I IFNs. These

cytokines activate a common receptor complex composed of 2 major subunits, IFNAR-1 and IFNAR-2.[34,35] IFNAR-1 is considered the signaling subunit, as it is absolutely required for signal transduction. There are 3 forms of IFNAR-2, which are differentially spliced products of the same gene, eg, the soluble (IFNAR-2a), short (IFNAR-2b), and long (IFNAR-2c) form.6,[36-38] The IFNAR-2c and IFNAR-1 subunits constitute the predominantly active form of the type I IFN receptor complex. IFNAR-2c is capable of binding ligand, but with a lower affinity (20-fold less) than the dimeric IFN receptor complex itself.[39] Therefore, both receptor chains are required to form a high affinity-binding site and initiate signal transduction leading to the induction of IFN-responsive genes. The short form is able to bind type I IFNs but does not couple to signal transduction.[40] The soluble form may act as a regulator of free IFNs and, depending on concentration, leads to the neutralization or even enhancement of IFN bioactivity.[41,42]

In the present study, we compared the antitumor effects of IFN-α and IFN-β, as well as the mechanisms that are involved in the growth inhibition of 3 human pancreatic cancer cell lines (BxPC-3, MiaPaCa-2, and Panc-1). Moreover, for the first time, we evaluated the expression and the subcellular distribution of type I IFN-receptor subtypes in these cells. We found that IFN-β potently inhibits cell proliferation already at very low concentrations (5–10 IU/mL) in BxPC-3 and MiaPaCa-2. These concentrations can be achieved in vivo after subcutaneous administration of IFN-β.[43,44]

The direct antitumor effects of IFN-α and -β are associated with the induction of apoptosis and cell cycle arrest. In BxPC-3 and MiaPaCa-2, both cytokines are able to induce apoptosis, but the increase in DNA fragmentation after IFN-β treatment occurred earlier and was considerably more potent than after IFN-α treatment. Panc-1 was the most resistant cell line to both IFNs, showing a stimulation of apoptosis only at very high doses (IFN-α ≥10,000 IU/mL, IFN-β ≥500 IU/mL). In all 3 pancreatic cancer cell lines, both IFNs induce a significant accumulation of cells in S phase compared with the untreated control, suggestive of a cell cycle arrest in the late S phase. The S-phase block induced by IFN-β is more potent and earlier than that of IFN-α.

Quantitative RT-PCR study and immunocytochemical analysis demonstrate the presence of all type I IFN receptor subunit transcripts and proteins in BxPC-3, MiaPaCa-2, and Panc-1 cells. The high expression of IFNAR-1 and IFNAR-2c subunits in BxPC-3 could explain the major sensitivity of this cell line to IFN treatment. Indeed, as shown by Wagner et al,[45] increasing the cell surface levels of IFNAR2c in cancer cells enhances their sensitivity to the antiproliferative and apoptotic effects of type I IFNs. Moreover, long-term cultures of IFNAR1-deficient mouse embryonic fibroblasts, as well as IFN-β deficient cells, resulted in the formation of transformed colonies in vitro and the formation of tumors in nude mice. It is interesting to observe striking differences in the subcellular localization and distribution of IFNs receptor subunits, as determined by immunocytochemistry. In BxPC-3, the staining for the active subunits of IFN receptor (IFNAR-1 and IFNAR-2c) is mainly membranous, whereas in Panc-1 the expression of IFNAR-2c is preferentially in the cytoplasm. A potential explanation to clarify the antitumor activity of type I IFNs at high doses in Panc-1, where IFNAR-2c subunits is mainly detected in the cytoplasm, may be that immunohistochemistry is not sensitive enough to demonstrate very low quantities of IFN membrane receptors. In addition, it has been recently observed that IFNAR-1, which is mildly to moderately expressed in the plasma membrane of several Panc-1 cells, has an important role in antiproliferative activity modulated by type I IFNs.[46] These arguments may explain why, even in the presence of low membrane expression of IFN receptors, antiproliferative and pro-apoptotic effects of high concentrations of type I IFNs are observed in Panc-1 cells. In Panc-1, about 60% to 70% of the cells exhibit no detectable levels of IFNAR-2c. This heterogeneity in IFNAR-2c expression may provide an additional explanation for the low sensitivity of these cells to IFN-α and IFN-β treatment. In Panc-1, it is possible a selection of cell type during type I IFN treatment, with higher possibility to survive for IFNAR-2c negative cells. Summarizing, these data suggest that the high sensitivity of BxPC-3 to IFNs treatment could be related to the strong expression of IFNAR-1 and IFNAR-2c and the main membranous localization, whereas the low expression, cytoplasmic localization, and heterogeneous staining of IFNAR-2c in Panc-1 could explain the relative resistance of these cells to IFN treatment. This is the first study, as far as we know, showing the importance of expression, distribution, and localization of type I IFN receptor subtypes in the

modulation of response to IFN treatment in pancreatic cancer. Our data also suggest that a careful evaluation of both active IFNAR subtypes in pancreatic cancer is required before treatment with type I IFNs is considered.

Although IFN-α and IFN-β interact with the same receptor, the induction of a differential response can be explained by the diversity in the structure between both cytokines,[47,48] generating different interactions and affinities for the related receptor. Indeed, IFN-β has a higher binding affinity (10-fold) than IFN-α.10 However, this cannot completely explain the difference in potency of cell growth inhibition between both cytokines, particularly in BxPC-3, where the IC50 for IFN-β is 40 times lower than that of IFN-α. Differences in the interaction of these IFNs with their receptors could be also involved. Both IFNs induce tyrosine phosphorylation of the receptor subunits; IFN-β, but not IFN-α, induces the association of IFNAR-1 and IFNAR-2c chains, indicating that the specificity of signaling for distinct type I IFN subtypes is established by differential conformation of the receptor complex.[40,49]

Conclusion

This study shows that IFN-β is significantly more effective than IFN-α in inducing cell growth inhibition in pancreatic cancer because it induces a more potent and early cell cycle arrest and apoptosis activation compared with IFN-α. Considering that IFN-β stimulates apoptosis already at very low dose, this cytokine could be a more promising agent than IFN-α for the treatment of human pancreatic cancer, particularly in tumors with a high expression of IFNAR-1 and IFNAR-2c, which is supporting its use in future clinical investigation. In addition, there is clear in vitro evidence that differential expression levels and distribution of the IFNs receptor subunits play a role in the regulation of the response to type I IFNs therapy in pancreatic cancer. Future studies should investigate in vivo whether the intensity, subcellular localization, and distribution of IFNAR-1 and IFNAR-2c at immunohistochemistry may predict the response to therapy with both IFN-α and IFN-β in pancreatic cancer.

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Chapter 8

Effects of Interferons α/β on the Proliferation of Human Micro- and Macrovascular Endothelial Cells

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Abstract

Introduction: Synthetic interferons (IFNs) are used in the treatment of several types of cancer. In addition to an antitumor effect, IFNs show antiangiogenic activity. The aim of this study was to investigate the effects of IFN-α and IFN-β on human micro- and macrovascular endothelial cells in vitro.

Methods: By immunohistochemical staining and quantitative reverse transcriptase (RT)-polymerase chain reaction, we studied expression of type I IFN receptors. We evaluated the effects of IFN-α and IFN-β on the proliferation (DNA content), apoptosis (DNA fragmentation by enzyme-linked immunosorbent assay), and cell cycle distribution (flow-cytometric analysis) of human micro vascular lung endothelial cells (HMVEC-L) and human umbilical cord endothelial cells (HUVEC).

Results: HUVEC and HMVEC-L cells show comparable expression level of the distinct IFN receptor subtypes. Proliferation of HMVEC-L and HUVEC was inhibited by IFN-β (the half maximal inhibitory concentration [IC50] was 60 and 90 IU/mL, respectively), but not by IFN-α at a dose up to 1,000 IU/mL. An interesting and unexpected observation was an inhibition of apoptosis by IFN-β. After 72 h of treatment with IFN-β. Cell cycle inhibition occurs in late S-phase in both cell lines.

Conclusion: Only IFN-β, not IFN-α (10–1,000 IU/mL), has an inhibitory activity on endothelial cell proliferation. Surprisingly, apoptosis was decreased by IFN treatment, whereas inhibition of proliferation is caused by cell cycle arrest in late S-phase.

Introduction

Interferons (IFNs) were first discovered in 1956 by Alick Isaacs and Jean Lindemann[1] as cytokines released by virus-infected cells inducing endonucleases and inhibitors of protein synthesis in neighbouring cells creating an antiviral state. Synthetic IFNs are currently used in the treatment of several types of cancer, multiple sclerosis, and viral infections such as hepatitis B/C and severe acute respiratory syndrome. IFNs are highly species specific and consist of 3 families of glycoproteins: type 1–3 IFNs. The type 1 family includes the IFN-α (leukocyte IFN), IFN-β (fibroblast IFN), IFN-ω, and IFN-τ subtypes. Type 2 (IFN-γ) and the recently discovered type 3 IFNs (IFN-λ)[2] will not be further discussed in this article. Thirteen forms of IFN-α and only one of IFN-β are known to exist[3]. The type 1–3 IFNs act through separate membrane receptors that have distinct properties and induce different responses. The type 1 receptor consists of 2 subunits, AR1 and AR2. AR2 is known to have 3 splice variants: long, short, and soluble forms. Only one form of AR1 has been identified[4]. Binding of IFNs to their receptor causes heterodimerization of the 2 receptor subunits, AR1 and AR2c, the long form splice variant[5]. One of the 2 membrane-bound variants of AR2, the short form AR2b, is able to bind IFNs but does not couple to signal transduction because it lacks the signal transducing tail of AR2c[4,6]. This soluble AR2a may act as regulator and stabilizer of free IFNs[7,8]. After heterodimerization, Tyk 2 and Jak 1 associate with the receptor complex and form a docking site for STATS 1 and 2. These STATS then translocate to the nucleus were they activate transcription of IFN-stimulated genes[9-11]. The list of IFN-stimulated genes is still increasing and currently over 300 genes have been identified[12]. IFN-α and IFN-β induce different subsets of genes in fibrosarcoma cells[13]; however, no differences were found in endothelial cells[14].

IFNs have known antitumor effects[11] and we recently demonstrated a more potent in vitro antitumor activity of IFN-β compared to IFN-α in adrenal, pancreatic, and neuroendocrine tumors[15-17] In vivo studies show growth inhibition of several types of cancer by IFNs. Inability to respond to endogenous IFN-α and IFN-β in type 1 receptor-deficient mice accelerated development, tumorigenity, and metastasis of cutaneous neoplasms[18]. In hepatocellular carcinoma grown in

nude mice, IFN-α inhibits metastasis and recurrence after curative resection by inhibition of angiogenesis mediated through downregulation of vascular endothelial growth factor[19]. Gene-therapy-delivered IFN-β is able to reduce invasiveness and angiogenic potential of prostate and renal cell cancer in vivo[20,21].

It is clear that endothelial cells play a critical role in tumor growth, since the size to which a tumor can grow is limited to the diffussional capacity of the tissue for oxygen and nutrients. An angiogenic switch[22] is necessary to expand beyond this point. Proliferation of endothelial cells is thought to be an essential step in this process of angiogenesis; therefore, inhibition of proliferation may result in antiangiogenic effects[23-25]. In vitro both stimulatory[26-28] and inhibitory[14,29-35], effects of type 1 IFNs on endothelial proliferation have been reported. Remarkably, IFN-β, potentially the most potent subtype[15-17] has not been studied extensively. Therefore, in the current study a comparison is made between the anti-proliferative effects of IFN-α and IFN-β on micro- and macro vascular endothelial cells in *vitro*.

Materials and Methods

Cell culture

Human umbilical cord endothelial cells (HUVEC) and human microvascular lung endothelial cells (HMVEC-L) were supplied by Clonetics-Cambrex Bioscienceä (Walkersville), and cultured in EBM-2MV® medium supplied by the same company. All supplements (human epidermal growth factor [hEGF], Hydrocortisone, vascular endothelial growth factor, human fibroblast growth factor [hFGF-B], Long R3-IGF-1, Ascorbic Acid, GA-1000 [antibiotics], and fetal bovine serum 5%) were added to the medium. The cultures were grown at 37°C and 5% CO_2. Standard culture instructions supplied by Clonetics were applied. Cells were passaged by trypsinization (trypsin EDTA [0.05%/0.53 mM]; Gibco, Grand Island, Canada). Before plating, the cells were counted microscopically using a standard heamocytometer. Trypan Blue staining was used to assess cell viability, which always exceeded 95%. HMVEC-L were used only from passages 3–6 and HUVEC passages 3–7.

Drugs and reagents

Intron-A (IFN-α-2b), REBIF (Recombinant IFN-β 1a), and Poly I:C were obtained from Schering-Plough Corporation (Utrecht, The Netherlands), Serono (Rockland, MA), and Sigma-Aldrich (Leiden, The Netherlands), respectively. All compounds were stored at −20°C, and the stock solution was constituted in distilled water according to the manufacturer's instructions.

Quantitative reverse transcriptase-polymerase chain reaction

Expression of receptors (AR1, AR2 total, the short-form AR2b, and the long-form AR2c) and housekeeping gene hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA was evaluated by quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) in HUVEC and HMVEC-L cells as published previously[15]. In addition, IFN-β primer and probe sequences were purchased from Biosource (Nivelles, Belgium): forward, 5′-CAGCAATTTTCAGTGTCAGAAGCT-3′; reverse, 5′-TTCATCCTGTCCTTGAGGCAG-3′; probe 5′-FAM TGTGGCAATTGAATGGGA GGCTTGAAT-TAMRA-3′

The detection of HPRT mRNA was used for normalization of mRNA levels. Expression of AR2a mRNA, the soluble form of AR2 subunit, was determined indirectly by subtracting AR2b and AR2c from AR2 total. Several controls were included in the RT-PCR experiments. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of HPRT and type I IFN receptors, human cDNA [from BON cells] was amplified in parallel with the cDNA samples.

Immunocytochemistry

HUVEC and HMVEC-L cells were grown on Thermanox® coverslips (Nunc, Naperville, IL) fixed with acetone and subsequently incubated for 30 min at room temperature with antibodies to human AR-1 (rabbit polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA) or AR-2c (monoclonal antibody, Dr

E. Croze, Berlex Biosciences, Richmond, CA) subunits. Standard streptavidin-biotinylated alkaline phosphatase (IL Immunologic, Duiven, The Netherlands) was used according to the manufacturer's recommendations to observe the bound antibodies. Negative controls for the immunohistochemistry were included by omission of the primary antibody.

Proliferation assay

After plating of cells (20,000 cells per well HMVEC-L and 10,000 cells per well HUVEC experiments) on 24-well plates (Corning, Schiphol-Rijk, The Netherlands), the plates were left in an incubator for 24 h for the cells to adhere to the bottom of the wells. After refreshing the medium, compounds were added. The medium was refreshed at day 3 and compounds added again. After 6 days the cells were harvested and DNA contents were measured. Total DNA contents, representative for the number of cells, were measured using the Hoechst 33258 reagent as published previously[36].

DNA fragmentation assay

After plating 100,000 cells/well for HUVEC and 200,000 for HMVEC-L on a 24-well plate, cells were left overnight to adhere. After refreshing the medium, compounds were added. After incubation for 24 h DNA fragmentation (apoptosis) was assessed using the Roche Cell Death Detection ELISA Plus® (Penzberg, Germany). This assay is based on a quantitative sandwich-enzyme-immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates, typical for apoptosis. The standard protocol supplied by the manufacturer was used. By both light microscopy and measurement of DNA contents, we confirmed that cell numbers were not significantly affected during these experiments.

IFN-β expression

In baseline conditions IFN-β expression is low but detectable. To explore the possible induction of IFN-β expression, cells were plated and refreshed as described for the apoptosis assay. Poly I:C, double-stranded RNA, a potent activator of IFN-β expression[37], was added at a concentration of 100 µg/mL and samples were collected after 2 and 4 h as described in the Quantitative RT-PCR section.

Cell cycle analysis by fluorescence-activated cell sorting

Cells ($0.8–2 \times 10^6$) were plated in 75 cm2 flasks. After 3 days, the medium was changed with a fresh medium (control group) or with a fresh medium plus IFN-α or IFN-β at the concentration of 1,000 IU/mL. After 3 days of incubation (with a confluence of about 60%–70%), cells were harvested by gentle trypsinization and prepared for propidium iodide (Sigma-Aldrich) staining as published previously[15]. The stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) using CellQuest Pro Software.

Statistical analysis

All experiments were performed in quadruplicate, except for RT-PCR and fluorescence-activated cell sorting (FACS), which were performed in duplicate, and at least twice with comparable results. For statistical analysis, GraphPad Prism™ 4.0 (GraphPad Software, San Diego, CA) was used. One-way analysis of variance, followed by multiple comparison Tukey test, was used to determine statistical significance, defined as $P < 0.05$. All data are shown as mean ± standard error of the mean. Statistical significance is marked with an asterisk. Bars represent means, and whiskers, standard error of the mean.

Results

Receptors

Expression of AR1 and all the variants of AR2 mRNA have been evaluated by quantitative RT-PCR, as shown in Fig. 1A. In both cell lines, receptor subtypes AR1 and AR2 a/b/c have comparable distribution; overall expression levels are not significantly different between cell lines ($P > 0.05$ for all subtypes). Immunostaining of both cell lines shows the presence of AR1 and AR2c on endothelial cells (Fig. 1B–G). These 2 subtypes jointly represent the active signal transducing complex. Both cell lines show a comparable staining and distribution of receptors: whereas AR1 staining is diffuse and membrane bound and/or cytoplasmic, AR2 staining is granular and appears to be more cytoplasmic than membrane bound.

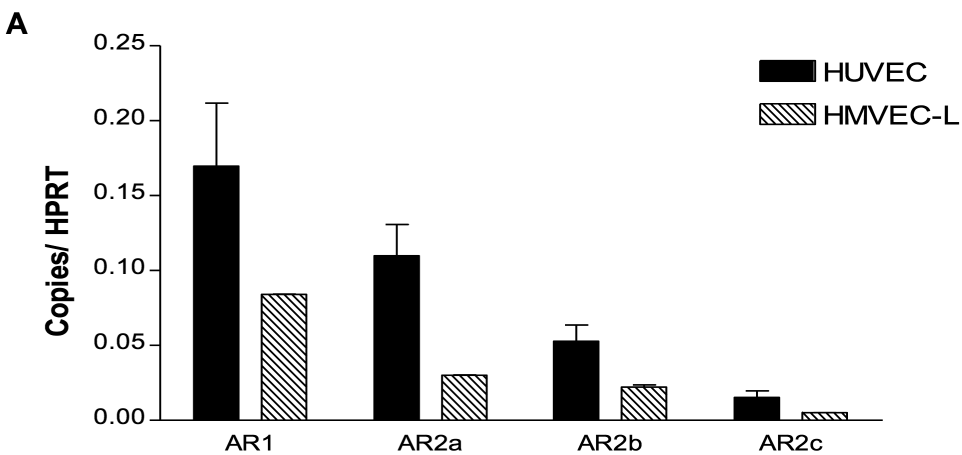
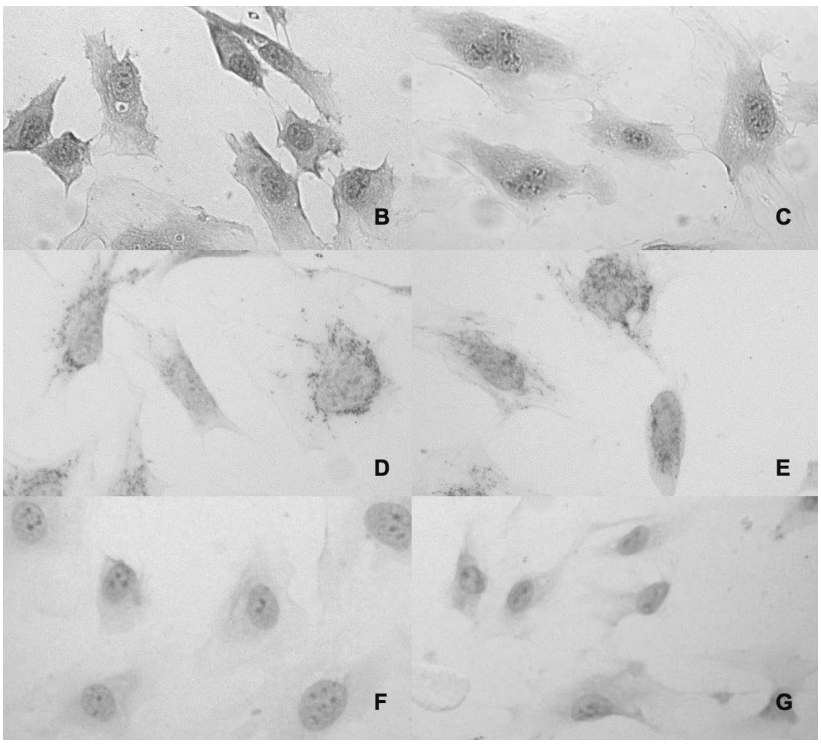


Figure 1: A: Expression of AR1, AR2a/b/c mRNA (Copies/ HPRT) ($p>0.05$ for all). Immunostaining: B: HUVEC AR1, C: HMVEC-L AR1, D: HUVEC AR2c, E: HMVEC-L AR2c, F: HUVEC negative control, G: HMVEC-L negative control

Endothelial cell proliferation



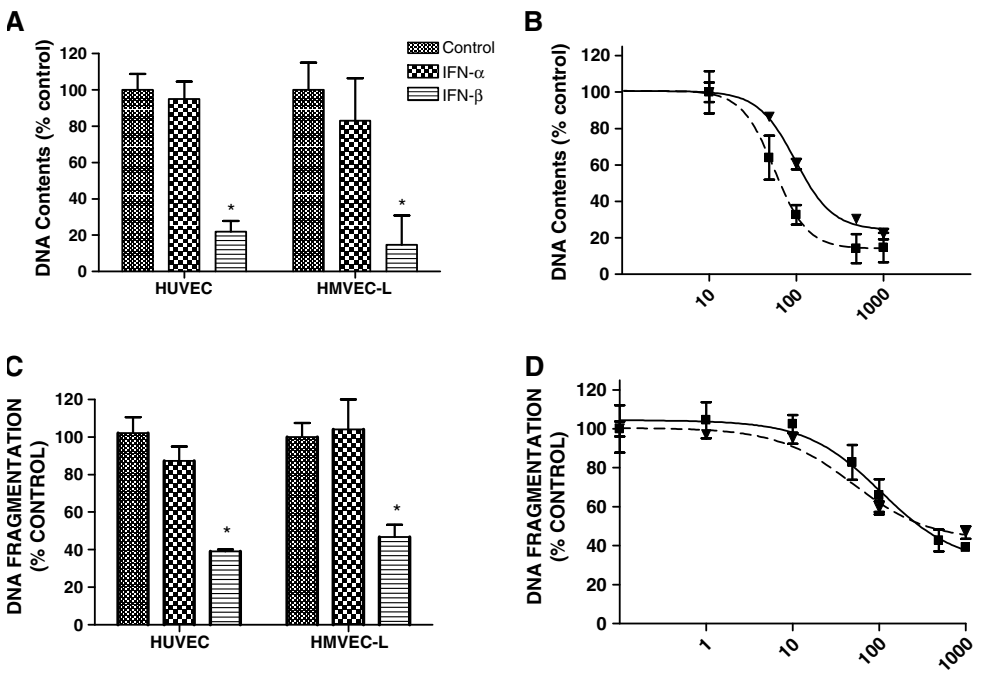
While IFN-α (10–1,000 IU/mL) does not induce any effect on the proliferation of HUVEC and HMVEC-L, IFN-β (10–1,000 IU/mL) treatment shows a statistically significant cell growth inhibition in both cell lines (Fig. 2A, B) in a dose-dependent manner [HMVEC-L: the half maximal inhibitory concentration [IC_{50}] = 60 IU/mL (95% confidence interval:38–84); HUVEC: 90 IU/mL (78–122) $P > 0.05$]. Population doubling times for HUVEC are 41 h, and 81 h after IFN-β treatment (1,000 IU/mL), for HMVEC-L 81 and 384 h, respectively.

DNA fragmentation

Stimulation of apoptosis by IFN-β has been reported to be one of the main mechanisms involved in the inhibition of proliferation, particularly in cancer cells[15,17]. Therefore, we measured DNA fragmentation after 24 h of IFN treatment (Fig. 2C, D). Surprisingly, IFN-β inhibits DNA fragmentation in a dose-dependent manner [HMVEC-L: IC_{50} = 51 IU/mL (95% confidence interval: 26–97);

HUVEC: 105 IU/mL (39–281) at 1,000 IU $P < 0.001$ for both cell lines]. IFN-α did not have a significant effect on apoptosis at a similar dose ($P > 0.05$). After 24 h of incubation with IFN-α or IFN-β, there was no change in cell number (DNA measurement; data not shown)

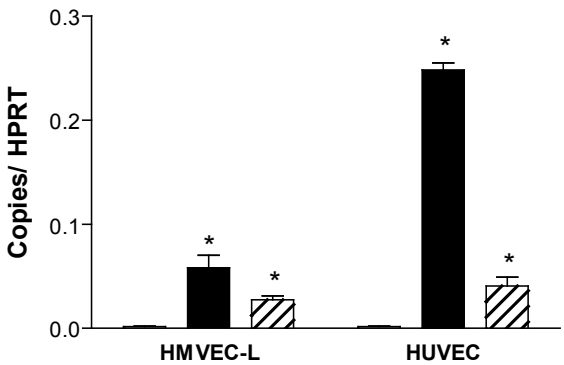
Figure 2: A: Effects of IFN-α and IFN-β treatment on proliferation after 6 days. (Dotted line: HMVEC-L) B: Dose dependent effects of IFN-β treatment on proliferation after 6 days. C: Effects of IFN-α and IFN-β treatment on DNA fragmentation (apoptosis) after 24 hours. D: Dose dependent effects of IFN-β treatment on DNA fragmentation after 24 hours. (* $p < 0.05$)



IFN-β expression

Figure 3 shows the IFN-β expression levels with and without poly I:C treatment. Poly I:C binds to a toll-like receptor (TLR-3) and is a known inducer of an antiviral response in cells[38]. Under baseline conditions, IFN-β expression is very low but detectable. After 2 h of Poly I:C treatment there was a dramatic increase of IFN-β mRNA (HUVEC: $P < 0.001$ and HMVEC-L: $P < 0.05$), followed by a decrease after 4 h (HUVEC: $P < 0.05$ and HMVEC-L: $P > 0.05$). The increase of IFN-β mRNA during stimulation was significantly higher in HUVEC than in HMVEC ($P < 0.001$).

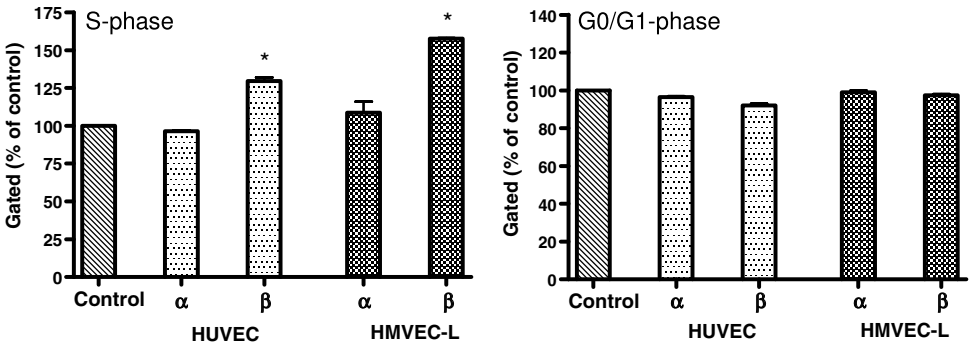
Figure 3: IFN-β expression levels with and without poly I:C treatment



Cell cycle effects

The effects of treatment with IFN-α (1,000 IU/mL) and IFN-β (1,000 IU/mL) on the cell cycle phase distribution have been evaluated in HUVEC and HMVEC-L cells. While IFN-α does not affect S-phase phase cell counts after 72 h of incubation, IFN-β induces a significant accumulation of both cell lines in S phase compared to the control. The proportion of cells in G0-G1-phase is not modified by either treatment in HUVEC and HMVEC-L.

Figure 4: Cell-cycle effects of Type 1 interferons



Discussion

Endothelial cell proliferation is an essential step in angiogenesis. Therefore, the inhibition of this process may result in antiangiogenic effects[23-25]. In literature there are contradictory results about the effects of IFN-α and IFN-β on endothelial cells, showing both inhibitory[14,31,33,35,39] and stimulatory effects[26-28] on proliferation. These apparent discrepancies between the different studies may be related to the culture conditions, concentrations, and the synthetic IFN subtypes used. The aim of the present study was to evaluate the antiproliferative effects of IFN treatment on endothelial cells, and especially the differences between IFN-α and IFN-β subtypes. Parallel to HUVEC, the most commonly used model for endothelial cells, we also studied HMVEC-L because of their adult microvascular origin, possibly resembling tumor endothelial cells more accurately. A completely different endothelial cell model next to HUVEC adds to the biological validity of our results.

For the first time, type I IFN receptors are identified immunohistochemically on both HUVEC and HMVEC-L. Both cell lines have similar expression of these receptors. Functional activity of the receptor complex is shown by the response to IFN treatment. Both cell lines can promptly express IFN-β when stimulated by poly I:C. Presumably this is similar to in vivo pathophysiological circumstances, such as during viral infections.

We assessed growth of endothelial cells after IFN treatment. Both cell lines HUVEC and HMVEC-L show a potent inhibition of proliferation by IFN-β. IFN-α is not able to inhibit the growth of endothelial cells at a concentration ranging from 10 to 1,000 IU/mL. Additionally, cell cycle analysis by FACS shows an increase in S-phase after 72 h of IFN-β treatment, for both cell lines (Fig. 4). This represents a cell-cycle block in late S-phase, as has been reported before in several tumor cell lines as well[15-17]. IFN-α does not induce any effect on cell cycle distribution at this dose range. The number of cells in G1/0 seems unaffected after IFN-β treatment, considering that we showed that the apoptosis, mitosis, and growth rates are decreased in the treatment group (Fig. 2). The increase in S-phase can be compensated by a decrease in the aforementioned phases. Possibly, only after prolonged incubation, a decrease in G0/1 may be observed. Since our cells were not synchronised, more and more cells arrive at S-phase and are blocked during IFN-β treatment, leading to accumulation in S- and decrease in G1/0 and other phases.

Both HUVEC and HMVEC-L show a dose-dependent decrease in apoptosis after IFN-β treatment. This was unexpected and in contrast with our findings in tumor cells[15-17]. An analogous effect was noted by Pammer et al.[35] They found that IFN-α could prevent apoptosis induced by serum starvation in HUVEC, using an identical cell death detection ELISA. They also studied endothelial cell apoptosis under normal culture conditions, without observing a significant effect on apoptosis by IFN-α.

Wada and others[39] recently showed a moderate inhibitory effect of high-dose IFN-α (10,000 IU) on cell growth of HUVEC cells. The following were observed: (1) a slight suppression of apoptosis was reported during IFN-α treatment (apoptosis in control versus IFN-α: 11 versus 7.6%); (2) IFN-α induced accumulation of HUVEC cells in S-phase after 24 h of incubation. In these experiments a less proliferative culture medium was used possibly leading to lower dose thresholds for IFN effects. However, a proliferative medium may more accurately resemble a highly angiogenic tumor environment. Inadvertently, HMVEC-L must be cultured in EBM-2MV® medium; in a less proliferative environment these cells do not proliferate consistently. For matters of comparability it was necessary to culture HUVEC in this medium as well.

As mentioned above, Wada and others[39] reported a moderate inhibitory effect on cell proliferation using high-dose of IFN-α (10,000 IU/mL) and only a slight effect with lower doses. We did not observe a significant inhibitory effect on cell proliferation at 1,000 IU/mL of IFN-α, but we did not evaluate the activity of this cytokine at higher supra-clinical dose. In addition, we observed a potent inhibitory effect on HUVEC and HMVEC-L cell proliferation after incubation with IFN-β. This could partially be explained by the fact that although IFN-α and IFN-β interact with the same receptor, IFN-β has a binding affinity ~10-fold higher than IFN-α[40].

Apoptosis and the cell cycle are considered to be intimately coupled41. Indeed both processes share many of the controlling pathways. However, according to the classical proliferation–survival model[42] apoptosis should increase when proliferation is inhibited. Our results show a contradictory relation. In a study by Tanaka[43] it was shown that IFNs are key mediators of apoptosis in virus-infected cells. Healthy cells could not be induced to undergo apoptosis unless they were co-stimulated. Similar co-stimulatory effects were seen in adhesion molecule expression (inter-cellular adhesion molecule 1 [ICAM-1] and vascular cell adhesion molecule 1 [VCAM-1]) after IFN-β treatment; endothelial cells were only stimulated by IFN-β to express these molecules when co-stimulated by tumor necrosis factor-α[44]. Takaoka and Taniguchi[45] propose that even under physiological circumstances, IFNs play a critical role in "revving up" cells, making them more able to respond quickly to external and internal signalling. In pathophysiological circumstances, tumor and virus-infected cells have intrinsic and extrinsic co-stimuli; this makes them susceptible to the cytotoxic effects of IFN-β [43,45,46]. HUVEC and HMVEC-L may lack these co-stimuli.

In conclusion, there is clear evidence that IFN-β inhibits proliferation of endothelial cells. The main cause of growth inhibition is a cell-cycle block in S-phase. This may partially explain the antiangiogenic effects as seen in vivo after IFN treatment, although paracrine effects also appear to be involved[19,39]. This hypothesis should be further strengthened by future in vivo or in vitro studies (such as endothelial cell migration or tube formation on matrigel). Furthermore, IFN treatment is not directly cytotoxic or pro-apoptotic for endothelial cells, in contrast to tumor cells. [15-17]. Currently, IFN-α is the most widely used type 1 IFN

in clinical-oncological treatment protocols[11,47]. Since our data suggest a more potent effect of IFN-β treatment, further in vivo and clinical research on this point is warranted.

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Chapter 9

Summary and Future Perspectives

Pancreatic and non-pancreatic periampullary cancer

The results of our randomised trial on adjuvant intra-arterial chemoradiation (Chapter 4) emphasize that PDAC and NPPC have a different biological behaviour and require a different adjuvant approach. Adjuvant intra-arterial chemoradiation and radiotherapy appeared beneficial for patients suffering from NPCC by reducing the occurrence liver metastases, but did not lead to prolonged survival in PDAC. The observation that we are dealing with different diseases despite the adjacent anatomical location is supported by several other trials on adjuvant therapy, such as the CONKO-1 and ESPAC trials. This is the most convincing argument that these cancers are different, at least in response to therapy, probably also in origin and biomolecular composition.

This hypothesis is maintained throughout this thesis. And indeed in Chapter 5 we show that there are differences in growthfactor receptor expression: The EGFR is almost exclusively expressed on PDAC, and may serve as an identifier of this tumor type. Most interesting was the negative influence on survival. The overexpression of the EGFR may be a additional factor in the malignant potential of these tumors. Other prognostic factors include differentiation grade, perineural invasion and lymphnode status. For most patients suffering from PDAC and to a lesser extent NPPC recurrence is inevitable. PDAC is almost always a systemic disease and not (only) a radical resection, but more important, the biology of the tumor itself determines the time to recurrence, effectiveness of adjuvant therapy and survival (Chapter 2). In the ESPAC-1 trial[1], patients with a R-1 resection had less benefit of adjuvant therapy compared to patients who had undergone a R-0 resection. The authors concluded that R-1 status is not an indicator of poor surgery, but of agressive tumor biology and consequently poor response to chemotherapy. A similar observation was made by Kooby et al.[2]. During pancreatoduodenectomy, re-resection to achieve a definitive negative resectionmargin after a positive pancreatic neck margin at frozen section, does not improve overall survival. Again the positive margin status indicates an aggressive and infiltrative tumor and a surgeon cannot change this biology by resecting another centimeter of pancreatic tissue.

In Chapter 6 we attempted to separate PDAC and NPPC based on differential genetic imbalances. By means of an arrayCGH we evaluated genetic gains and losses. Many gains and losses were present in both tumor types, this pattern was very variable throughout both PDAC and NPPC, indicating the unstable and heterogenous character of these cancers. We found four CGH clones to be differentially gained or lost between PDAC and NPPC. We could not reliably differentiate between our clinically relevant groups of PDAC and NPPC by these underlying genetic imballances. We also did not find any other sub-classification by hierachical clustering. A drawback of an aCGH is the relatively macro-genetic method to evaluate these tumors and perhaps we will be able to find these subgroups using gene- or RNA- level methods. These methods are also able to detect single mutations and epigenetic factors that may influence expression and functionality of specific genetic areas. For now we must conclude that they are very much alike in their genetic re-arrangements.

Currently, there is no validated molecular or biological classification for NPPC or PDAC and therefore we are forced to classify by means of macroscopic and microscopic histological assessment. Standard microscopic histological assessment by itself cannot differentiate between NPPC or PDAC. Two histological subtypes, intestinal and pancreatobiliary differentiation can be identified in both PDAC and NPPC by microscopic assessment. In addition, several immunohistochemical markers such as CK20, CDX2 and MUC1 are available. Pancreatobiliary differentiation may indicate more aggressive biological behavior. When NPPCs with pancreatobiliary differentiation are compared to PDACs, survival is similar, whereas intestinal differentiation in PDAC is associated with a longer survival. Although pancreatobiliary and intestinal differentiation may divide NPPCs and PDACs in two distinct and probably relevant groups, it cannot be used to differentiate between PDAC and NPPC since both NPPC and PDAC can have intestinal or pancreatobiliary differentiation. However for future studies, simple and widely available staining of intestinal markers such as CK20 and CDX2 should be added to identify and possibly stratify for these two most relevant histo-molecular subgroups. Future studies probably should also incorporate analysis of other molecular or genetic markers (or at least collect and store tissue samples), although a clear direction, based on the current literature, is difficult to give.

The key point is that the origin of a “periampullary” cancer is an essential part of the multidisciplinary discussion whether a patient may benefit from adjuvant therapy in terms of disease free and overall survival. We conclude that determination of the origin of a periampullary cancer (NPPC subtypes and PDAC) is essential for optimal clinical management and should therefore be systematically incorporated in clinical practice and future studies.

Interferons in the treatment of pancreatic cancer

In Chapter 7 we showed the results of type 1 interferon treatment on pancreatic cancer cell-lines and in Chapter 8 on human endothelial cells. From these experiments it is clear that interferon β is much more potent than α. Furthermore, some cancer cell lines are more sensitive to this treatment than others. Cancer cells undergo apoptosis and this anti-tumor effect is the main cause of growth inhibition. In (healthy) endothelial cells arrest in S-phase and not apoptosis (this is actually inhibited) is the main cause of growth inhibition. It is very interesting to hypothesize why these responses are so completely different. Type 1 Interferons play a critical role in “revving up” cells, making them more able to respond quickly to external and internal signalling. In pathophysiological circumstances, tumor and virus-infected cells have intrinsic and extrinsic co-stimuli; this makes them susceptible to the cytotoxic effects of interfero α and β. HUVEC and HMVEC-L may lack these co-stimuli and are therefore simply “halted” in S-phase to wait for further signalling.Interferon α was clinically evaluated as adjuvant therapy after resection for PDAC in a randomized trial[3]. Disappointingly, as an single addition to adjuvant fluorouracil based chemotherapy there was no clinical benefit. Several aspects should however be considered and that is were the last two chapters of this thesis add to the “interferons in pancreatic cancer” discussion. There is a different potential for interferon β, compared to α. The major clinical drawback is that interferon β is pharmacologically very instable and will probably not reach a pancreatic tumor at significant levels after percutaneous injection. We speculate that other ways of delivering interferon β to a tumor (glycosylated, gene therapy, direct injection, coated beads etc.) could overcome these issues.

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Chapter 10

Summary in Dutch

Samenvatting

Pancreas en periampullaire carcinomen die niet van het pancreas uitgaan

De langetermijnsresultaten van onze gerandomiseerde studie naar intra-arteriële chemotherapie laten zien dat het pancreascarcinoom (PDAC) en en periampullaire carcinomen die niet van het pancreas uitgaan (NPPC) een verschillende respons op adjuvante therapie hebben. In deze studie was adjuvante intra-arteriële chemotherapie van voordeel voor patiënten die leden aan NPPC door effectieve en langdurige remming van het ontstaan van levermetastasen. Voor patiënten die leden aan PDAC was het echter niet van voordeel. Deze observatie ondersteunt het idee dat we te maken hebben met twee verschillende groepen tumoren. Dit wordt verder ondersteund door enkele andere studies, zoals de CONKO-1[1] en ESPAC[2] trials. Het verschillend biologisch gedrag is het belangrijkste argument dat PDAC en NPPC verschillend zijn, klinisch qua respons op therapie maar waarschijnlijk ook in origine en biomoleculaire samenstelling.

Deze hypothese is de rode draad door deze thesis. En in hoofdstuk 4 laten we zien dat er inderdaad een verschil tussen NPPC en PDAC is in expressie van de epidermale groeifactor. De epidermale groeifactor komt vrijwel exclusief voor op PDAC en lijkt een negatieve invloed op de prognose te hebben. Andere factoren die een relatie hebben met de overleving zijn differentiatiegraad, perineurale invasie en lymfklierstatus.

Voor de meeste patienten die lijden aan PDAC en in mindere mate voor hen die lijden aan NPPC is een recidief na in opzet curatieve chirurgie onvermijdelijk. PDAC is een systemische ziekte en niet een “radicale” resectie, maar de biologie van de tumor bepalen de uitkomst. In een sub-analyse van de ESPAC-1 studie werd aangetoond dat tumoren met een positieve snijrand een slechtere respons op chemotherapie hadden[3]. De auteurs concludeerden dat de marge status een indicator van slechte biologie is en niet van slechte chirurgie. Kooby et al. zagen een vergelijkbaar fenomeen: als het pancreasresectievlak middels per-operatieve vriescoupe positief was en er een extra stuk gereseceerd werd waardoor deze

negatief werd, verbeterde de prognose niet[4]. Wederom bepaalde de biologie van de tumor de uitkomst en niet de marge.

In hoofdstuk 5 hebben we geprobeerd NPPC en PDAC te scheiden op basis van hun aCGH profiel. We vonden veel afwijkingen met aCGH, maar konden geen subgroepen onderscheiden. Slechts vier CGH-clones waren verschillend in aantal tussen beide groepen. Hierarchische clustering en primary component analyse waren niet gecorreleerd aan de klinische parameters en leverden ook geen andere subgroepen op. Een bekend nadeel van CGH is dat er naar relatief grote gebieden in het genoom wordt gekeken. Wellicht zijn er met gevoeliger technieken of expressie geörienteerde arrays wel verschillen aan te tonen. Voor nu moeten we op basis van onze data concluderen dat er opvallend weinig verschillen zijn.

Op dit moment is er geen eenduidige moleculaire of biologische classificerende methode beschikbaar om NPPC en PDAC te onderscheiden. We zijn dus afhankelijk van macroscopie en microscopie. Histologisch zijn PDAC en NPPC echter niet te onderscheiden. Er zijn echter wel twee histologische subtypen van PDAC en NPPC: intestinaal en pancreato-biliair. Daarnaast zijn er ook markers zoals CK20, MUC1 en CDX2 waarmee hiertussen een onderscheid kan worden gemaakt. Pancreato-biliaire differentiatie is geassocieerd met een slechtere prognose. NPPC met pancreato-biliaire differentiatie heeft net zo een slechte overleving als PDAC. Deze subtypen verdelen deze tumoren in twee relevante groepen, maar komen zowel in NPPC als PDAC voor en kunen hierin dus niet helpen bij het maken van een onderscheid. Voor volgende gerandomiseerde studies is het bijhouden van deze verdeling wel te adviseren, ondersteund door kleuringen met CDX2 en CK20.

Het belangrijkste punt is dat de origine van een ”periampullaire tumor” een essentieel onderdeel moet zijn bij de multidisciplinaire bespreking of een patient wel of geen baat heeft bij (neo) adjuvante chemotherapie. Het maken van een onderscheid tussen NPPC en PDAC is belangrijk voor het maken van een behandelplan en moet dus onderdeel vormen van de dagelijkse klinische praktijk en toekomstige studies.

Interferon en de behandeling van pancreascarcinoom

In hoofdstuk 6 hebben we de resultaten laten zien van behandeling met type 1 interferons op pancreas- en endotheelcellen in vitro. Uit deze experimenten blijkt dat interferon β een veel sterker effect heeft dan α. Daarnaast zijn sommige tumorcellen gevoeliger dan andere. Tumorcellen gaan in apoptose tijdens de behandeling met interferon en dat is de belangrijkste oorzaak van de groeiremming. Gezonde endotheelcellen gaan niet in apoptose (dit wordt zelfs geremd) maar worden geremd in de late S-fase van de celcyclus. Dit is een interessant fenomeen om over te speculeren. Type 1 inteferons spelen een grote rol bij het aanjagen van cellen als er gevaar dreigt, bijvoorbeeld een virusinfectie, zodat ze snel kunnen reageren op de buitenwereld. In pathologische omstandigheden (bijvoorbeeld in tumorcellen) krijgen cellen secundaire signalen van buiten en binnen en dat maakt ze wellicht gevoeliger voor de apoptose inductie door interferon. HUVEC en HMVEC-L cellen hebben deze signalen niet, en zouden daarom alleen worden gestopt in S-fase, in afwachting van co-stimuli.

Interferon α is in klinische gerandomiseerde studies onderzocht als adjuvante behandeling in combinatie met chemotherapie na resectie voor PDAC. Helaas was de toxiciteit flink en gaf het geen overlevingswinst[5]. Een aantal aspecten moet echter overwogen worden, en dat is waar de laatste twee hoofdstukken van deze thesis iets kunnen toevoegen aan de “interferon en pancreascarcinoom” discussie. Er is namelijk een groot verschil tussen de effectiviteit van interferon α en β. Daartentegen is een groot nadeel van interferon β ten opzichte van α is dat het farmacologisch erg instabiel is en waarschijnlijk geen adequate spiegels in de doelorganen te verkrijgen zijn met subcutane injectie. Het toedienen van interferon β op een andere wijze, bijvoorbeeld in gepegyleerde vorm, gentherapie, gecoate beads of directe injectie in de tumor, zou deze problemen kunnen verhelpen.

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Chapter 11

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Dankwoord

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Chapter 12

List of publications

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Chapter 13

Curriculum Vitae Auctoris

Curriculum Vitae Auctoris

Joris Erdmann werd op 12 april 1977 geboren in Zwolle en groeide, na met zijn ouders een paar jaar in Londen te hebben gewoond, op in het Gelderse dorpje Heerde. Na het behalen van het eindexamen aan het Gymnasium Celeanum in Zwolle verhuisde hij in 1997 naar Rotterdam en ging daar Beleid en Management voor de Gezondheidszorg en later Economie studeren. Na eindelijk te zijn ingeloot begon hij in 2000 aan zijn studie Geneeskunde aan de Erasmus Universiteit. Tijdens zijn studie werkte hij als student mee aan het wetenschappelijk onderzoek bij de afdeling Heelkunde van het Erasmus MC. Hier werd de basis gelegd voor zijn huidige promotieonderzoek. Daarnaast werkte hij in het studententeam “Les Forgerons” op de spoedeisende hulp van het Ikazia Ziekenhuis. Eind 2006 behaalde hij Cum Laude het artsexamen en werd begin 2007 aangenomen voor de opleiding Heelkunde. Deze opleiding begon hij de eerste twee jaar in het Erasmus MC, en daarna tot en met het vijfde jaar in het IJsselland Ziekenhuis te Capelle a/d IJssel. Zijn laatste opleidingsjaar heeft hij zich in het Erasmus MC volledig toegelegd op de pancreaschirurgie onder leiding van Prof. dr. C.H.J. van Eijck. Na het afronden van de opleiding tot - en certificering als oncologisch en gastro-intestinaal chirurg vervolgde hij zijn opleiding in 2013-2014 met het Groningse fellowship “Hepato-Pancreato-Biliary Surgery and Liver Transplantation”. Na afronding van dit fellowship werd hij eind 2014 aangenomen als staflid Hepato-Pancreato-Biliaire Chirurgie en Levertransplantatie in het UMCG.

Chapter 14

Supplementary Information

PhD Portfolio

Summary of PhD training and teaching

Name PhD student:	**Erasmus MC Department:**
Joris I. Erdmann	Surgery
PhD period:	**Promotor:**
2007-2015	Prof.C.H.J. van Eijck, MD PhD

Supervisor:
N.F.M.Kok, MD PhD

1. PhD training:

Courses	*Year*	*Workload (ECTS)*
BROK (Basiscursus Regelgeving Klinisch Onderzoek)	2015	1.0
ESSU course Milaan	2013	1.0
Desiderius School:		
Module Ziekenhuismanagement	2012	1.0
Module Gezondheidsrecht	2012	1.0
Masterclass Laparoscopic Liver Surgery	2012	1.0
CASH cursus	2010-2012	1.2
OCEH (Elancourt/ Milaan)	2010-2011	3.0
LISA courses	2008-2011	1.0
Presentations:		
International		6.0
National		1.5
Conferences:		
National Surgical Conferences	2008-2015	4.0
International HPB and Surgical	2008-2015	16.0

2. Teaching and lecturing:

Courses	*Year*	*Workload (ECTS)*
Nurses/Medical students/(Surgical) residents		
Erasmus MC	2008-2013	10.0
UMCG	2013-2015	8.0

TNM-classificatie voor pancreascarcinoom (UICC 2009).

0	Tis,N0,M0
IA	T1,N0,M0
IB	T2,N0,M0
IIA	T3,N0,M0
IIB	T1-3,N1,M0
III	T4,N0-1,M0
IV	T1-4,N0-1,M1

Tis, carcinoma in situ
T1, tumor beperkt tot de pancreas, maximaal 2 cm. in grootste diameter
T2, tumor beperkt tot de pancreas, groter dan 2 cm. in grootste diameter
T3, directe uitbreiding buiten de pancreas, maar zonder betrokkenheid van de truncus coeliacus of de arteria mesenterica superior. T4, betrokkenheid van de truncus coeliacus of de arteria mesenterica superior.

N0, geen regionale lymfkliermetastasen aangetoond
N1, metastase(n) in regionale lymfklier(en)

TNM classificatie voor distale extrahepatische galwegen (UICC 2009).

0	Tis,N0,M0
IA	T1,N0,M0
IB	T2,N0,M0
IIA	T3,N0,M0
IIB	T1-3,N1,M0
III	T4,N0-1,M0
IV	T1-4,N0-1,M1

Tis, carcinoma in situ
T1, tumor beperkt tot de galgang
T2, tumor is door de wand van de galgang gegroeid
T3, tumor is uitgebreid naar de galblaas, lever, pancreas, duodenum, of andere aangrenzende organen
T4, betrokkenheid van de truncus coeliacus of de arteria mesenterica superior

N0, geen regionale lymfkliermetastase(n) aangetoond
N1, metastase(n) in regionale lymfklieren

TNM classificatie voor de papil van Vater (UICC 2009).

0	Tis,N0,M0
IA	T1,N0,M0
IB	T2,N0,M0
IIA	T3,N0,M0
IIB	T1-3,N1,M0
III	T4,N0-1,M0
IV	T1-4,N0-1,M1

Tis, carcinoma in situ

T1, tumor beperkt tot de papil van Vater of de sfincter van Oddi

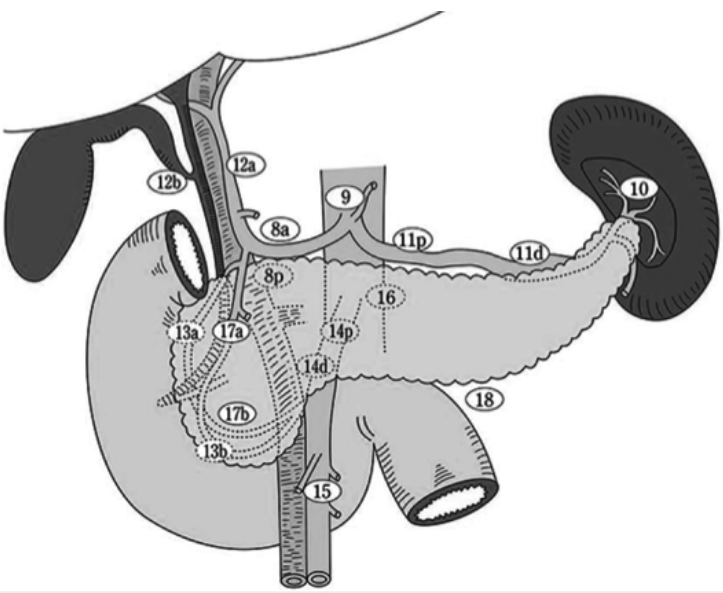
T2, tumor groeit in de duodenumwand

T3, tumor is uitgebreid naar hetpancreas

T4, tumor is uitgebreid naar de peripancreatische weke delen of naar andere aangrenzende organen of Structuren

N0, geen regionale lymfkliermetastase(n) aangetoond

N1, metastase(n) in regionale lymfklieren



Lymphnode stations according to the JPS clasification (Tumors of the Pancreas , Atlas of Tumor Pathology Series 4, with permission)