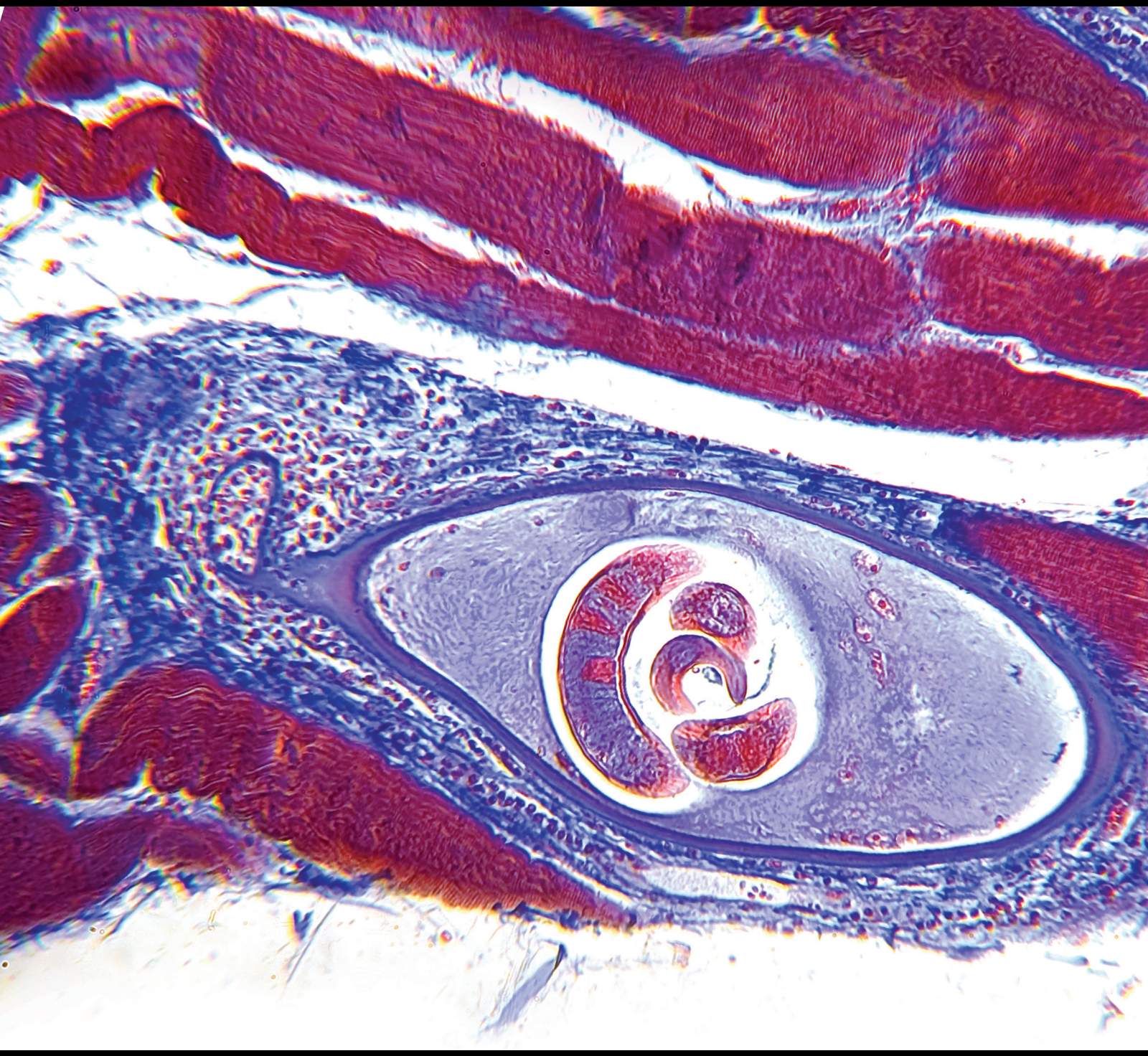
Gastroenterology Research and Practice

Pancreatic Cancer

Guest Editors: Niccola Funel, Marco Del Chiaro, Djuna L. Cahen, and Johanna Laukkarinen



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Editorial
Pancreatic Cancer

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Pancreatic tumors are challenging diseases. Pancreatic adenocarcinoma (PDAC), in particular, is associated with significant morbidity and mortality. Another group of exocrine tumors, intraductal papillary mucinous neoplasia (IPMNs), represents the paradigm of progression of malignancy and their connection and differentiation with respect to pancreatic ductal adenocarcinoma (PDAC) which is still complicated. In this special issue, we would like to offer readers an overview of some important aspects for the two most representative exocrine tumors as IPMN and PDAC. The original communications within this special issue fall into four different categories: pancreatic surgery, management of IPMN patients, treatment of pancreatic tumors, and, last but not least, some important aspects of basic science in PDAC.

Surgery. Curative resection is considered the only potential for cure in pancreatic cancer. Complete macroscopic tumor resection is perhaps the most relevant predictor of long-term survival in PDAC. Locally advanced pancreatic tumors can involve vascular structures and adjacent organs, and thus vein resections and even resections of additional organs may be needed to achieve the goal. Improving surgical methods to achieve this goal is crucial in the treatment of this disease.

In pancreatic surgery, postoperative pancreatic fistula (POPF) remains the most challenging complication after resections, whether pancreatoduodenectomy or distal. POPFs are contributing significantly to prolonged hospitalization and mortality. Several studies using various anastomotic and sealing techniques have been studied to reduce the amount of POPFs. Some previous investigations have shown that pancreatic trauma and the following inflammation are preceding postoperative complications and they should be avoided. Patients with normal, acinar-cell rich pancreas are in higher risk to develop POPF. One promising technique for pancreatoduodenectomy is the Finnish binding pancreaticojejunal anastomosis (FBPJ), where the pancreatic trauma is minimized by avoiding sutures running through the pancreatic tissue. The preliminary previous studies with this technique have shown reduced amount of POPF after pancreatoduodenectomy.

IPMN Management. Intraductal papillary mucinous neoplasms of the pancreas (IPMNs) are a high prevalence neoplasm of the pancreas. IPMNs can progress from adenoma to invasive cancer through a process very similar to the one of the colonic polyps. At the moment the management of pancreatic IPMN is suggested by the Guidelines of the International Association of Pancreatology and by the European Guidelines for Cystic Tumors of the Pancreas. However, till now, the available evidence about the diagnosis and treatment of those tumors is quite low and the clinical decisions are made on the basis of expert consensus (more than guidelines) and local expertise. Even though IPMNs represent in one side an opportunity to prevent pancreas cancer (through

an early detection and treatment of precancerous lesions), considering the high risk associated with pancreatic surgery, there is a potential risk to overtreat patients that might never develop cancer. Even the decision making in the intraoperative management of IPMNs is complicated: the extent of resection, the role of parenchyma sparing procedure, and the role and significance of margins analysis are argument under investigations and have not been yet defined.

To make the overall picture even more complicated, the accuracy of the current imaging modalities is reported very low even in high volume centers. In a recent series the overall accuracy in defining preoperative diagnosis in cystic tumors of the pancreas was inferior to 70% and even the use of endoscopic ultrasound plus FNA was not able to increase the results. More large studies are needed in order to better understand the natural history of these tumors, to discriminate the ones that can progress to cancer from the ones with low aggressive behavior. At the same time, new insight into the field of diagnostic is necessary in order to increase the accuracy of imaging modalities. For the reasons mentioned above and for the tremendously high prevalence, IPMNs represent today maybe the most challenging area of interest in pancreatology, a great opportunity to reduce the pancreas cancer mortality, but also one of the most dangerous clinical areas.

Pancreatic Cancer Treatments. Pancreatic adenocarcinoma is one of the most deadly cancers, with an overall 5-year survival of 5%. Complete surgical resection provides the only chance for cure. Unfortunately, most patients are diagnosed with locally advanced or metastatic disease. Chemotherapy, with and without radiation, has been investigated in both neoadjuvant and postoperative settings. At present, multimodality therapy seems to be the future direction. However, the sequence of surgery, chemotherapy, and radiation remains to be determined. In the review of this special issue, the authors examined available data on neoadjuvant treatment in resectable patients and in patients with borderline resectable or locally advanced disease.

Of course, the need for new therapies is undisputed. One of the latest local treatments is high-intensity focused ultrasound (HIFU), a noninvasive and safe technique to ablate solid tumors. In this special issue, the authors have reviewed all 3022 cases, described in the literature, with respect to safety and efficacy.

Basic Science of PDAC. In the papers published in this special issue, the authors treated three molecular aspects regarding the PDAC: indicator tryptase, ATP-binding cassette (ABC) transporters, and micro-RNA (miRNA) expression. These molecules are involved in the progression, chemoresistance, and survival of PDAC patients, respectively. In particular, M. Ammendola et al. have shown the role of tryptase in PDAC which is associated with mast cells to increase the microvascular density (MVD) in tissue PDAC. This could play an important role in vascularization, the absorption of drugs, and chemoresistance of PDAC. In fact, drug chemoresistance of PDAC cells is recognized as the primary cause of failure of chemotherapy. Although biochemical behaviors, including low drug concentration in the tumor, may contribute to clinical resistance, different types of molecules, including ATP-binding cassette transporters, are the main actors in the extrusion of drugs in different tumors. In particular, some single nucleotide polymorphisms (SNPs) of the three most important family genes of ABC (ABCB1, ABCC1, and ABCG2) are associated with a lower risk of developing pancreatic cancer and an increased sensitivity to gemcitabine than other haplotypes. Finally, the most important epigenetic factors, such as miRNAs, were confirmed to be "micromolecules," regulating "the great effects." Their deregulation modulates several important processes in PDAC, including differentiation, tumor progression, and epithelial-mesenchymal transition (EMT). Nevertheless, miRNAs affect overall survival and chemoresistance of PDAC patients. Aberrant expression of three most important miRNAs (miR-21, miR-155, and miR-101) is strongly associated with PDAC, IPMN, and nonmalignant lesions, respectively.

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Review Article

Extent of Surgery and Implications of Transection Margin Status after Resection of IPMNs

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Appropriate surgical strategies for management of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas are a matter of debate. Preoperative and intraoperative evaluation of malignant potential of IPMN and of patient's comorbidities is of paramount importance to balance potential complications of surgery with tumors' risk of being or becoming malignant; the decision about the extent of pancreatic resection and the eventual total pancreatectomy needs to be determined on individual basis. The analysis of frozen-section margin of pancreas during operation is mandatory. The goal should be the complete resection of IPMN reaching negative margin, although there is still no agreed definition of "negative margin." Of note, the presence of deepithelization is often wrongly interpreted as absence of neoplasia. Management of resection margin status and stratification of surveillance of the remnant pancreas, based on characteristics of primary tumour, are of crucial importance in the management of IPMNs in order to decrease the risk of tumor recurrence after resection. Although risk of local and distant recurrence for invasive IPMNs is increased even in case of total pancreatectomy, also local recurrence after complete resection of noninvasive IPMNs is not negligible. Therefore, a long-term/life-time follow-up monitoring is of paramount importance to detect eventual recurrences.

1. Introduction

Described for the first time by Ohhashi and Murayama [1] in 1982 as a specific tumor-entity distinct from mucinous cystic neoplasms (MCNs) and ductal adenocarcinoma (PDA), intraductal papillary mucinous neoplasms (IPMNs) are increasingly being recognized. With the widespread use of cross-sectional imaging, IPMNs are nowadays frequently detected in asymptomatic individuals [2, 3]. Of note, in high-volume centers for pancreatic surgery, IPMNs represent one of the most common indications for pancreatectomy [4].

Histologically, IPMNs are mucin-producing neoplasms, arising from the main and/or secondary pancreatic ducts, with prominent intraductal growth and frequent papillary architecture [5]. The World Health Organization divided IPMNs into different entities, based on the involvement of pancreatic ductal system [5, 6]: *main-duct type* (MD-IPMN), when the tumor involves only the main pancreatic duct; *branch-duct type* (BD-IPMN), when the tumour involves only branch-ducts, with no macroscopic or microscopic involvement of the main pancreatic duct; and *mixed type*, when the neoplasia involves macroscopically and/or microscopically both the main pancreatic duct and its side branches. Because of their similar clinic-pathological characteristics and behaviour, mixed IPMNs are grouped with main-duct IPMNs in the patient's management [3]. On the basis of the degree of cytoarchitectural dysplasia [7, 8], IPMNs are associated with a spectrum of dysplastic changes of the epithelium, ranging from low-grade dysplasia (IPMN adenoma), intermediate-grade dysplasia (IPMN borderline), and high-grade dysplasia (IPMN with *carcinoma in situ)* that are considered to be noninvasive and IPMN with invasive carcinoma (invasive IPMN).

Furthermore, multifocal lesions can be found both in MD-, combined-, and BD-IPMNs [9]. BD-IPMNs present multifocal lesions in about half cases [3, 9]. Main pancreatic duct (MPD) can be entirely involved by the tumor that may extend along it or by synchronous skip lesions that are present in around 20% of the patients [10, 11]. In the light of the heterogeneity of this group of tumors and of their different risk of malignancy, it is important both preoperative and intraoperative evaluation of the malignant potential of the IPMN, in order to balance the risk of complications of pancreatic surgery with the IPMN risk of being or becoming malignant over time. The aim of this review is to analyze the factors influencing the extent of surgical resection and the implications of transection margin status during pancreatectomy for tumor recurrence and patient survival after surgery.

2. Management of IPMN

Guidelines based on experts' opinion and on the available evidence from the literature have been put forward by the International Association of Pancreatology in 2006 and revised in 2012 [6, 12] and by the European Study Group on Cystic Tumours of the Pancreas [13]. However, management of IPMNs is complex and several areas of uncertainty still remain in their treatment, both for the indications for surgical resection and for the surgical management of these patients.

2.1. Indications for Surgical Resection of Main Duct and Mixed IPMNs. In the light of the high frequency of malignancy and invasive carcinoma, even in patients without symptoms [14] or lacking radiological malignant parameters (enhanced solid component, MPD size of ≥ 10 mm) [12, 15], surgical resection is strongly recommended for all surgically fit patients with a clinic-radiologic diagnosis of MD- and combined-IPMNs [12], in order to achieve complete removal of the tumor with a negative margin. In consideration of tumor site and its extension along MPD, pancreaticoduodenectomy (PD), left pancreatectomy, or total pancreatectomy (TP) with splenectomy (LP) with lymph node dissection represent the treatment of choice [12].

2.2. Indications for Surgical Resection of Branch Duct IPMNs. Considering that the frequency of malignancy in resected BD-IPMN is around 25% and that these lesions mainly affect elderly patients with comorbidities increasing the surgical risk, a conservative management with continuous clinic-radiologic followup is recommended for the patients without risk factors for malignancy. Recent guidelines put forward by the International Association of Pancreatology (IAP) [12] identify (i) some "worrisome features" on imaging that include cyst of ≥ 3 cm, thickened enhanced cyst walls, MPD size of 5–9 mm, nonenhanced mural nodules, abrupt change in the MPD caliber with distal pancreatic atrophy, and lymphadenopathy and (ii) some "high-risk stigmata" for indication to resect (obstructive jaundice, enhanced solid component, MPD size of ≥ 10 mm).

In general, "high-risk stigmata" represent always a clear indication for surgical resection. On the other hand, BD-IPMNs without "high-risk stigmata" can undergo surveillance, with more strict follow-up timing for those patients with "worrisome features." In a significant number of patients multiple BD-IPMNs can be detected (multifocal disease) [12, 16]. More than 50% of the patients with BD-IPMN present a multifocal disease with multiple BD-IPMNs along the gland [6]. There are no evidences of a higher rate of malignancy in multifocal BD-IPMNs [17]. In these cases, the approach follows the same criteria for the unifocal BD-IPMNs: when indication for surgical resection is present, standard segmental pancreatectomy can be performed if the lesions are confined to a single pancreatic region; otherwise, an extended resection up to a total pancreatectomy should be considered [12, 18, 19]. However, if the entire gland is involved, smaller lesions without malignancy-related features can be left behind and, instead of total pancreatectomy, a partial pancreatectomy should be performed [17].

2.3. Preoperative Planning of the Extent of Surgical Resection. In order to follow a correct oncologic approach, surgical treatment of an IPMN should be preceded by a careful study of the tumor topography and extent and of its possible signs of malignancy. Both computer tomography (CT) scan and magnetic resonance cholangiopancreatography (MRCP) are useful tools to assess the tumor and its relationship to nearby structures including peripancreatic vessels and to detect lymphadenopathy and/or distant metastases. Endoscopic ultrasound (EUS) is also a useful diagnostic tool to demonstrate thickness of the walls, mural nodules, and adjacent masses. EUS should be also combined with fine-needle aspiration in order to evaluate both cytology and pancreatic enzymes and tumoral markers (i.e., CEA and CA19-9). Moreover, ERCP can be used to demonstrate dilated pancreatic ducts and defects caused by mucin plugs or intraluminal neoplastic nodules; however, the slight risk of pancreatitis associated with this procedure must be considered.

Preoperative imaging is yet not always reliable to define the real entity of the tumor and its extension along the gland. So, at the operating theatre, the aim of surgical resection is the eradication of IPMN, and the intraoperative examination of the transection margin is of paramount importance to determine the necessity to proceed with a further pancreatic resection until the achievement of a resection margin free of epithelial atypia [20, 21], up to a possible total pancreatectomy [6, 9, 17, 20, 22, 23]. There is no agreement about the "right" extent of resection: some surgeons customarily resect all the gland involved by the tumor even if it results in total pancreatectomy and there is evidence of low-grade dysplasia, in order to avoid recurrence [21, 24]. On the other hand, an anatomic partial pancreatectomy, to preserve pancreatic parenchyma and to prevent metabolic consequences, is recommended by others [9, 25–27], thus stopping the resection once no high-grade dysplasia is present.

2.4. Parenchyma-Sparing Pancreatectomy. Parenchyma-sparing resections of the pancreas include enucleation, middle

pancreatectomy (MP), and middle-preserving pancreatectomy [28–31]. In order to decrease the risk of development of exocrine/endocrine insufficiency, some patients may benefit from these "atypical" resections [31, 32].

2.4.1. Enucleation. It has been proposed for "low-risk" BD-IPMN [31]. However, the great majority of low-risk BD-IPMNs can be safely managed nonoperatively, while in "high-risk" BD-IPMNs a formal pancreatectomy should be performed. Moreover, in patients with a suspected BD-IPMN at preoperative imaging undergoing enucleation, a proper histological examination of the connection with the MPD cannot be made, and a microscopic involvement of the MPD (combined IPMN) cannot be excluded. In the cases reported in the literature, no patient treated with enucleation developed tumor recurrence, but again, most of these patients had benign BD-IPMNs that could be also managed nonoperatively. On the other hand, a higher fistula rate has been reported compared to standard resections [33].

2.4.2. Middle Pancreatectomy (MP). It can be performed for BD-IPMNs in the neck or proximal body of the pancreas without malignancy-related features but with an indication for surgical resection. In this case, after an accurate preoperative study of the lesion to exclude clinical and radiological signs of malignancy, intraoperative histological examination of the two resection margins is mandatory; if a frozen-section examination is positive for malignant disease, the operation must be converted in a standard pancreatectomy. Perioperative morbidity after MP is higher than that for standard pancreatectomy [34], but, considering the preservation of pancreatic function, MP is an effective alternative to formal pancreatic resection [35]. MP has been proposed also for MD- or combined-IPMN involving exclusively the neck of the pancreas. Again, the intraoperative examination of both the transection margins must be performed. However, the recurrence rate after MP for MD-IPMNs is significant (33%) [35], limiting the role of MP for these tumors.

2.4.3. Middle-Preserving Pancreatectomy. It can be an effective alternative to total pancreatectomy, decreasing the risk of pancreatic insufficiency, when the lesions involve all pancreas except the body, as it could happen in multifocal IPMNs. After an intraoperative ultrasound, pancreaticoduodenectomy and distal pancreatectomy with splenectomy are performed leaving the pancreatic body: the two section margins are sent for frozen-section examination [31]. If tumor involvement is present, total pancreatectomy should be performed.

3. Transection Margin Status

3.1. The Definition of "Positive" Margin. There is no consensus about the definition of "positive" resection margin in case of pancreatectomy for IPMNs, and the lack of a clear definition implies a great heterogeneity among different studies.

Some authors classify surgical margins in IPMN as "negative" in case of presence of normal epithelium or mucinous hyperplasia without dysplasia in the main duct and as "positive" in case of adenoma, borderline neoplasm, or carcinoma [17].

Other authors instead follow another classification presenting negative resection margin (with normal columnar epithelium or denuded), mucinous hyperplasia (pancreatic intraepithelial neoplasia Pan-IN 1A or 1B), or positive resection margin (dysplasia Pan-IN 2 or carcinoma Pan-IN 3) [9, 36].

Considering the degree of dysplasia, in some studies, the surgical margin is reported as negative if it presents normal epithelium or IPMN adenoma and as positive in case of moderate or severe dysplasia (borderline IPMN or carcinoma *in situ* IPMN) [37].

Otherwise, a lesion can be considered "significant," thus requiring additional resection, in presence of at least IPMN adenoma on the main duct or at least borderline IPMN on branch ducts [38].

One of the factors most clearly associated with recurrence is the presence of deepithelialization (denudation) at the resection margin that is so wrongly interpreted as an absence of neoplasia [20, 38]. The presence of denudation should routinely lead to an extension of surgical resection [38], since it is associated with an increased rate of recurrence [39].

During frozen section analysis of the resection margin, there is also the possibility to find incidental PanIN lesions that are not always simple to distinguish from IPMN extending into small ducts. They are both intraductal proliferations of mucin-producing cells that may possess various degrees of atypia and have the potential to progress to adenocarcinoma [7]. PanINs are usually incidental microscopic findings associated with smaller ducts [7, 40]. The authors who performed additional resection only for high-grade dysplasia or invasive carcinoma classify PanIN eventuality as "no significant dysplasia" at the margin [41].

3.2. Intraoperative Implications of Transection Margin Status. Intraoperative analysis of the transection margin during pancreatectomy for an IPMN is important but also controversial because of its implications. In case of an IPMN involving, at the preoperative imaging, only a segment of the pancreas, the chance that the radiological imaging could underestimate the real extent of ductal involvement makes mandatory an intraoperative frozen-section (FS) histological examination to define the appropriate cut line [20, 38, 42–44]. When the intent of the pancreatectomy is a curative resection, many authors emphasize the importance of obtaining a tumor-free surgical margin by FS analysis [21, 25, 45, 46].

IAP guidelines suggest that when adenoma (low-grade dysplasia) is present at the resection margin, no further resection is required because the risk of progression to cancer or local recurrence is minimal; instead, moderate- or high-grade dysplasia as well as invasive carcinoma at the FS requires an additional resection, up to a total pancreatectomy [12].

However, in the light of the different definitions of "positive" margins, in the literature the optimal surgical strategy remains controversial.

In the study of Couvelard et al. [38], the result of the FS analysis implies an extent of the resection in 30% of the patients, allowing an adequate resection in 97% of the cases; similar percentage of additional resection is presented by Salvia et al. (21%) [14].

The percentage of concordance between FS and definitive examination of the margins is different in the various studies but tends to be high: all the intraoperative diagnoses have been confirmed at the final pathologic analysis in the study of Salvia et al. [14]; moreover, high accuracy rate has been reported by Fujii et al. (99%) [47], Raut et al. (97%) [48], and Couvelard et al. (94%) [38]. Nevertheless, lower percentages of concordance have been reported in the series of White et al. (67%, with a positive predictive value of frozen section of 50% and a negative predictive value of 74%) [22] and in the study of Frankel et al. (57%, with a positive predictive value of 41.2% and a negative predictive value of 66.7%) [49]. The case of misdiagnosis presented by Raut et al. [48] concerns a pancreaticoduodenectomy for noninvasive IPMN: while the FS margin was interpreted as negative, the final pathology report revealed the presence of a microscopic focus of noninvasive IPMN. The patient did not undergo reresection and did not develop recurrent disease. Regarding the conflicting results in the study of Couvelard et al. [38], even if 9 cases of "underestimation" and 3 cases of "overestimation" by FS are reported, only 4 patients (3%) have had inadequate extent of the pancreatic resection, excessive in one case and insufficient in 3 cases, consisting in normal epithelium versus IPMN adenoma or borderline IPMN or noninvasive carcinoma in main duct at the definitive examination. In the paper further resections for these patients are not reported. As a general recommendation, surgical reexploration and pancreatic resection should be considered when malignancy is found at final pathology on the resection margin, given the high risk of recurrence in this setting. The possibility of performing a total pancreatectomy must be carefully discussed with the patient. If the patient refuses the reoperation, a very strict radiological followup is mandatory on a 3-4-month schedule. When benign IPMN is found at final pathology on the resection margin, long-term clinic-radiological followup is preferable to immediate reresection.

When we look at the data on total pancreatectomy, the procedure has been performed heterogeneously, ranging from 2.7% [22] to 23% [9].

The analysis of FS margin is indicated in all IPMNs, but its implications on MD-IPMN and BD-IPMN are different. In BD-IPMNs, it is important to analyze the resection margin in order to exclude the presence of the tumor, but extension of the surgical resection is uncommon [38]. In MD- and combined-IPMNs, instead, frozen-section margin analysis is of paramount importance because dilatation of the MPD and neoplasia of the duct lining are not always correlated. In fact, dilatation of MPD can be due only to pancreatitis and obstruction by mucus upstream or downstream from the tumor [38]. The task of the pathologist is to deem the question with the microscopic analysis of the margin.

Moreover, the risk of a positive margin seems to be correlated with the degree of IPMN dysplasia, being significantly higher in patients with moderate- or high-grade dysplasia (50%) than in patients with low-grade dysplasia (22%) [49].

3.3. Pancreatoscopy. Since IPMN can arise in multiple sites within the pancreas and preoperative imaging can be inadequate in detecting microscopic spread of cancerous lesions or skip lesions [37], it is fundamental to evaluate if FS margin analysis is the only factor to be considered in determining the extent of the resection.

The knowledge of skip lesions and the development of recurrence in patients with noninvasive IPMN and negative surgical margin suggest that the real extension of the IPMN involvement of the pancreatic gland can be difficult to predict.

Hara et al. [50] recommend the combination of peroral pancreatoscopy and intraductal ultrasonography for an improved differential diagnosis between malignant and benign IPMNs [9].

A further help in planning extent of the resection can be provided by pancreatoscopy with narrow band imaging (NBI) that is done using flexible pancreatoscope through the cut end of the duct at the surgical margin after partial pancreatectomy. Kaneko et al. [11] have reported the incidence of multicentric lesions as high as 20.8%, with high rates of sensitivity and specificity for the procedure. Intraoperative pancreatoscopy allows an accurate examination of the entire duct and NBI facilitates in better identification of the vascular pattern of the lesion. The intraoperative pancreatoscopy with NBI access to main duct seems to be more accurate than peroral pancreatoscopy [27].

In order to detect skip lesions and hence the real intraductal tumor extension, an intraoperative 2- or 3-segmental cytology of the pancreatic juice, in addition to frozen-section analysis, can be performed [51–53]. A single-lumen catheter is inserted across the cut surface in the main pancreatic duct of the cranial pancreas and a triple-lumen catheter is inserted into the caudal pancreas to obtain the pancreatic juice separately from each portion of the pancreatic head, body, and tail. After cytological analysis, segments with positive cytology should be additionally resected. In the study of Eguchi et al. [37], all patients with positive cytology and negative surgical margins had skip lesions in further resected specimens. After histological and cytological examinations, 42% of the patients required additional resection. No patient developed a recurrence in the remnant pancreas. However, although these data are promising, they need to be confirmed in larger cohorts of patients.

4. Recurrence and Survival after Surgical Resection

Tumor recurrence after pancreatic resection for IPMN can be classified as local, regional, or distant (metastatic). Local recurrence is defined as the presence of an IPMN in the pancreatic remnant after partial pancreatectomy [9]. Recurrence after resection of noninvasive IPMN may occur because of (1) a residual dysplastic tissue at the surgical margin, (2) a multicentric tumor with synchronous skip lesions in the remnant pancreas undetected during initial operation, or (3)

metachronous lesions that have developed in the remnant pancreas as a result of a neoplastic tendency to involve the entire gland (field defect) [9, 23, 42, 54].

Chari et al. [9] analyzed a group of 133 patients resected for noninvasive IPMN (73 patients) and invasive IPMN (40 patients) and proposed a correlation between recurrence/survival after surgical resection and the histology of the tumor. Eight percent of noninvasive IPMN showed recurrence after partial pancreatectomy, after a median followup of 37 months and none of the 13 patients who underwent total pancreatectomy had extrapancreatic recurrence. In invasive IPMNs, recurrences were similar both after partial pancreatectomy and total pancreatectomy (67% and 62%, resp.), and 91% of them occurred within 3 years from surgery. Five-year survival was higher in noninvasive (84.5%) than in invasive IPMNs (36%). 26% of the patients showed a recurrence in the pancreatic bed or in the remnant pancreas, whereas 74% had either a distant metastatic recurrence or both local and distant metastatic recurrence. The most common metastatic site was the liver (65%). In case of invasive IPMN, the presence of dysplasia at the margin was the only predictor of recurrence.

D'Angelica et al. [55] presented a series of 63 patients with IPMN surgically managed. Of these, 51% had resection margins involved with atypia or carcinoma *in situ*; however, the presence of mild- or borderline dysplasia or carcinoma *in situ* at the resection margin was not associated with a poor outcome. 23% of the resected patients developed a recurrent disease, with half of these in the first 2 years. The median time from surgery to recurrence was 20 months. The rate of disease specific outcomes did not differ among patients with and without positive margins. Disease-specific 5-year survival was 75%. Significant predictors of poor outcome included elevated serum total bilirubin, presence of invasive carcinoma with its extent and type (tubular versus colloid), lymph node metastases, and vascular and perineural invasion. These factors were all significantly associated with the recurrence of tumor, unlike the margin status that was not associated with disease recurrence. Then, early oncologic outcome was determined by the pathologic characteristics of the primary tumor and not by the resection margin status.

Falconi et al. [20] showed a local recurrence in 8% of 51 patients with IPMN treated by pancreatic resection. Mild to moderate dysplasia was present at the frozen-section margin in 20 specimens (41%) and carcinoma in one.

All the patients with recurrence underwent a second resection. The 3-year survival rate for benign IPMNs was 94% and 69% for malignant ones. In this paper, the importance of the presence of deepithelialization of the resection margin was highlighted; local recurrence in patients with eroded epithelium at the surgical margin must lead to considering deepithelialization of the margin as a "positive resection margin."

Frankel et al. [49], in a study with 192 patients undergoing resection of noninvasive IPMN, showed a recurrence of 21% at a median followup of 46 months. Ductal dysplasia at the final surgical margin was defined by the presence of IPMN or PanIN, regardless of the degree of dysplasia. 31% of the patients with margin dysplasia recurred, whereas, among patients without dysplasia, 13% presented recurrent disease. However, this was not associated with poor survival. Of note, tumor recurrence was not found at the level of the surgical margin but in the remnant pancreas, far from transection line. Dysplasia at the resection margin was associated with recurrence in the remnant gland, but not at the resection margin. According to the authors, this indicates that, albeit a positive margin is associated with recurrence, it is more likely a marker of diffuse ductal instability and not a local oncologic failure.

Fujii et al. [47] considered 103 cases of noninvasive IPMNs including *carcinoma in situ* (CIS). Recurrences were observed in 4.9% of the patients with benign IPMN and in 22.7% of the patients with CIS; none recurred at the resection margin, 9 recurred in the remnant pancreas, and 1 at the peritoneal surface, probably for the preoperative EUS-guided fine-needle aspiration biopsy. The presence of adenoma at the resection margin seems to have no influence on the outcome, because recurrence was diagnosed in 7.8% of adenoma-negative patients and in 10.7% of adenoma-positive patients and overall survival and recurrence-free survival were similar between the two groups.

Salvia et al. [14] analyzed 140 patients with MD-IPMN (with or without side branch involvement). The rate of recurrence after resection in the remnant pancreas was 7%; only one patient did not have invasive cancer as primary tumor. Patients with noninvasive IPMN had a 5- and 10-year cancer-specific survival of 100%, whereas for patients with invasive carcinoma 5- and 10-year survival was 60% and 50%, respectively. Among the 32 patients with a positive or indeterminate resection margin, 4 (8%) developed a late recurrence in the remaining pancreas.

Schnelldorfer et al. [56] analyzed 208 patients with IPMN; 58% of the invasive IPMNs recurred, whereas 10% of noninvasive IPMNs recurred after partial pancreatectomy and 0% after total pancreatectomy. Five-year survival in patients with noninvasive IPMN was 94%; instead, five-year survival in patients with invasive IPMN did not differ much from 5-year survival of a matched cohort with ductal adenocarcinoma (31% versus 24%).

In case of negative margin of resection, the median survival was 119 months and the 5-year survival rate was 77% and those were greater but not statistically significantly different from those of patients with "benign" positive margin (62 months and 52%). Otherwise, patients with malignant positive margin had the worse survival rate (median survival 11 months and 5-year survival rate 0%).

Sohn et al. [54], considering 136 pancreatic resections for patients with IPMNs, found an overall 5-year survival for patients with IPMN without invasive cancer of 77% and of 43% in patients with an invasive component.

There were no differences in survival between patients with different dysplasia in the primary tumor (adenoma, borderline neoplasms, and CIS) and neither comparing BD-IPMNs, MD-IPMNs, and combined variants.

White et al. [22], in a series of 78 patients resected for noninvasive IPMN, found that there was no significant difference in local recurrence rates between BD-IPMNs (7.9%) and MD-IPMNs (7.5%). Local recurrence was described in 7.7% of the patients at a median followup of 40 months,

Table 1: Characteristics of noninvasive resected IPMNs.

Author	Total *n* (%) noninvasive IPMN	Positive margin, %	Recurrence rate, %	5-year survival, %	Median follow-up, months
Chari et al., 2002 [9]	73 (65)	3.3	8	84.5	36
D'Angelica et al., 2004 [55]	32 (52)	51.6 (noninv + inv)	4.8	91	32
Falconi et al., 2001 [20]	32 (63)	36.7	8 (noninv + inv)	94 (3-year surv.)	15 (mean)
Frankel et al., 2013 [49]	192 (100)	45	21	32.3	46
Fujii et al., 2010 [47]	103 (72)	27.2	9.7	—	41
Salvia et al., 2004 [14]	72 (51)	22.2	1.4	100	31
Schnelldorfer et al., 2008 [56]	145 (70)	2.8	10	94	—
Sohn et al., 2004 [54]	84 (62)	24	8.3	77	24 (mean)
White et al., 2007 [22]	78 (100)	29.5	7.7	87	40

Table 2: Characteristics of invasive resected IPMNs.

Author	Total *n* (%) invasive IPMN	Positive margin, %	Recurrence rate, %	5-year Survival,%	Median follow-up, months
Chari et al., 2002 [9]	40 (35)	26	65	36	42
D'Angelica et al., 2004 [55]	30 (48)	51.6 (noninv + inv)	14.5	58	32
Falconi et al., 2001 [20]	19 (37)	79	8 (noninv + inv)	69 (3-year surv.)	15 (mean)
Salvia et al., 2004 [14]	58 (41)	27.6	12.1	60	31
Schnelldorfer et al., 2008 [56]	63 (30)	28.6	58	31	—
Sohn et al., 2004 [54]	52 (38)	38.5	—	43	24 (mean)

with a median interval of 22 months from the resection. Only 2% of the patients with negative margins recurred, whereas 17% with positive margins presented recurrence, and thus also the majority of patients with positive margin of resection did not develop local recurrence; anyhow, local recurrence free survival was significantly higher for patients with negative margin than for patients with positive margin. Regarding PanIN-1 or -2, no patients with these lesions at the resection margin presented recurrence. In case of borderline or *carcinoma in situ* IPMN as primary tumor, the percentage of positive margin for IPMN was higher than that in case of patients with adenoma (42% versus 9%, resp.). The estimated 5-year local recurrence-free survival for all patients resected was 87%.

Tables 1 and 2 summarize the main data of the abovementioned studies.

Data in Tables 1 and 2 show a difference in the behavior of invasive and noninvasive resected IPMNs. In case of noninvasive tumors, even if the percentage of positive margin is significantly different in the various studies, the percentage of recurrence rate and 5-year survival can be considered quite similar, that is, uncommon recurrence and good prognosis, regardless of the positivity of resection margin. Otherwise, when the resected tumors are invasive, data are significantly different in the studies, suggesting that other factors, in addiction to resection, determine the prognosis.

5. Followup

IPMN is often a slow-growing tumor, so recurrences, if any, can occur late after resection and might be underestimated after short-term followup [9, 57–59]. Therefore, long-term or, probably, life-time followup with surveillance imaging is required [9, 56]. However, there are no clear indications regarding the frequency, duration, or methods of postoperative surveillance in patients with resected noninvasive IPMNs [60, 61].

Patients with noninvasive IPMN after partial pancreatectomy with negative margins must be informed about the risk of recurrence and, in case of appearance of symptoms as abdominal pain, pancreatitis, jaundice, new-onset steatorrhea, or weight loss, they should be investigated promptly with a radiologic study (CT or MRI) [9].

The frequency of followup should be adjusted based on the risk of recurrence. Patients with IPM-adenoma, negative resection margin, and normal remnant pancreas on postoperative imaging can follow a yearly or biannual surveillance. For

patients with IPM-borderline or CIS and a positive resection margin or indeterminate cystic lesions in the remnant pancreas, the surveillance should be more frequent for the first 2 to 3 years (twice a year), when most of recurrences usually occur [12]. In any case, indefinite radiographic surveillance must be recommended, because recurrences might occur even more than 5 years after resection [9, 14, 22, 54].

For patients with invasive IPMN, the indicated followup should be identical to that for ductal adenocarcinoma with surveillance every six months [12].

It has been suggested that patients with IPMN present an increased risk of developing additional pancreatic and extrapancreatic malignancies [62–65], which may occur before, after, or concurrent with the diagnosis of IPMN. PDAC is detectable in 2–10% of patients with BD-IPMN, in separate regions from the IPMN [66, 67]. The most common extrapancreatic malignancies are gastric and colorectal cancers [41, 68–70]; therefore, surveillance for these secondary malignancies must be maintained in these patients [37, 70].

6. Conclusions

All these data confirm that resection is the treatment of choice for MD- and combined-IPMN and for BD-IPMN with high-risk stigmata. The analysis of frozen-section margin of pancreas during the operation is mandatory, and it plays a role in the proper management of MD- and combined-IPMNs. In case of invasive cancer or intermediate/high-grade dysplasia, an additional resection, up to eventual total pancreatectomy, should be performed.

The percentage of concordance between FS and definitive examination of the margins tends to be high in the various studies; anyway, in the literature, there are no guidelines for the event of a conflicting result between FS and definitive examination of the resection margin. At any rate, as general recommendation, surgical reexploration and pancreatic resection should be considered when malignancy is found at final pathology on the resection margin for the high risk of recurrence in this setting. The possibility of a total pancreatectomy must be carefully discussed with the patient. If the patient refuses the reoperation, a strict radiological followup every 3-4 months is recommended. When benign IPMN is found at final pathology on the resection margin, long-term clinical and radiological followup is preferable to immediate reoperation.

Recurrence after complete resection of noninvasive IPMNs is uncommon but not negligible, regardless of the degree of epithelial dysplasia in the neoplasm. There is an increased risk of local, regional, or distant metastatic recurrence for invasive IPMNs even in the setting of "curative" resection, usually within the first 3 years of resection. Liver is the most common site of metastatic recurrence, and total pancreatectomy does not prevent the recurrence of cancer. Furthermore, in the light of the possible severe metabolic consequences, prophylactic total pancreatectomy is not recommended in patients with apparently limited disease and negative margins. Total pancreatectomy should be strongly considered in young, fit patients with high-grade dysplasia (including at least Pan-IN 3) or invasive cancer at the resection margin. It is evident that the decision about the extent of pancreatic resection and the eventual total pancreatectomy needs to be determined on individual basis, considering the preoperative patient's status and the presence of comorbidities, symptoms, or an already existing insulin-dependent diabetes.

Of note, the presence of deepithelization is often considered as absence of neoplasia; however, the association of "denuded" epithelium at the resection margin with tumor recurrence suggests that it is wrongly interpreted as negative margin. In these cases, an extension of surgical resection is mandatory.

A critical point in the management of patients with IPMNs is the postoperative surveillance. Recurrence can be due to a multifocal disease, with a synchronous, undetected, IPMN present within the remnant pancreas or because of the development of a metachronous IPMN as a result of a widespread neoplastic field defect in the pancreatic ductal epithelium. Therefore, follow-up monitoring is of paramount importance to detect recurrence. Current guidelines recommend stratification of surveillance of the remnant pancreas based on characteristics of primary tumor. Patients with a high risk of recurrence should be assessed every 3–9 months with cross-sectional imaging, whereas low-risk patients may be screened annually/biannually. Since recurrences can occur even 10 years or more after resection, a long term/life-time followup after resection of IPMN is mandatory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Utilization of Imaging Features in the Management of Intraductal Papillary Mucinous Neoplasms

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Intraductal papillary mucinous neoplasms (IPMNs) represent a group of cystic pancreatic neoplasms with large range of clinical behaviours, ranging from low-grade dysplasia or borderline lesions to invasive carcinomas. They can be grouped into lesions originating from the main pancreatic duct, main duct IPMNs (MD-IPMNs), and lesions which arise from secondary branches of parenchyma, denominated branch-duct IPMNs (BD-IPMNs). Management of these cystic lesions is essentially based on clinical and radiological features. The latter have been very well described in the last fifteen years, with many studies published in literature showing the main radiological features of IPMNs. Currently, the goal of imaging modalities is to identify "high-risk stigmata" or "worrisome feature" in the evaluation of pancreatic cysts. Marked dilatation of the main duct (>1 cm), large size (3–5 cm), and intramural nodules have been associated with increased risk of degeneration. BD-IPMNs could be observed as microcystic or macrocystic in appearance, with or without communication with main duct. Their imaging features are frequently overlapped with cystic neoplasms. The risk of progression for secondary IPMNs is lower, and subsequently an imaging based follow-up is very often proposed for these lesions.

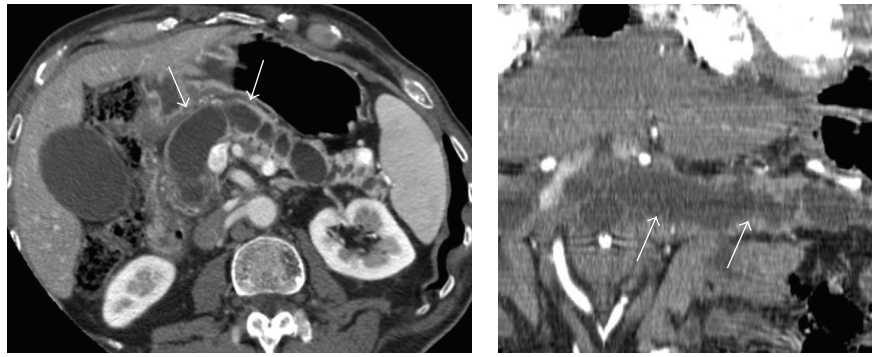
1. Introduction

Intraductal papillary mucinous neoplasms (IPMNs) are a subgroup of cystic pancreatic neoplasms, representing an estimated 0.5–9.8% of all pancreatic exocrine tumours [1, 2]. Their incidence has been modified in the last decade, due to the large amount of IPMNs occasionally reported after cross-sectional imaging [3, 4]. In the last 15 years also Salvia et al. confirmed the increase in frequency of IPMNs, with an incidence of disease ranging again from 0.5% up to 10% among all exocrine pancreatic tumours [5–8].

Initially, it was difficult to define their nosological entity and, consequently, these mucinous ductal tumours were known variously [1, 9]. Only in 1997 the term intraductal papillary mucinous neoplasms (IPMN) was introduced by the WHO (Word Health Organization) [1, 10]. The term refers to a group of pancreatic neoplasms originating—in papillary form—from the epithelium of the duct system and leading progressively to a dilatation of the duct, which progressively develops a cystic appearance.

As with many other cancers, the origin of IPMNs is still unknown. Since they were first reported, they have been associated with chronic pancreatitis.

In a recent multicentre control-case study published, some clinical conditions have been associated with the development of IPMN, including diabetes (particularly cases associated with insulin assumption), chronic pancreatitis, and a family history of pancreatic ductal adenocarcinoma [11].



(a) (b)

Figure 1: CT postcontrast examination in a patient who was suffering from jaundice and abdominal pain: axial images after contrast administration (a) and curved-MPR images (b). White arrows in (a) and (b) show a marked dilatation of the entire main pancreatic duct, from the head to the tail of the gland, associated with subtotal parenchymal atrophy. No dilatation of secondary branches was observed, and radiological diagnosis of MD-IPMN was formulated. The high degree of main pancreatic duct dilatation (>1 cm) was considered as high-risk stigmata and required surgical treatment. In addition, white arrows show mild wall enhancement. Final diagnosis of invasive cancer (adenocarcinoma) in IPMN was reported.

The natural history of small pancreatic cysts is not yet clearly understood. According to their biological behaviour, the WHO classification system currently separates IPMNs into

(i) benign (intraductal papillary mucinous adenoma)

(ii) borderline (intraductal papillary mucinous tumors with moderate dysplasia)

(iii) malignant (intraductal papillary mucinous carcinoma, noninvasive or invasive).

In fact, IPMNs display a spectrum of cytoarchitectural atypia, ranging from none to borderline to marked and can also be associated with invasive carcinoma [12]. Similarly to the mucinous cystic neoplasms and the pancreatic intraepithelial neoplasia (PanIN), IPMNs are currently considered precursors and precancerotic lesions of the pancreas [13]. The transformation from a benign into a malignant histologic type may take several years (approximately 5 years) and this event is not observed in all cases [14].

Cystic pancreatic neoplasms include a large spectrum of lesions with different radiological appearance [1, 3, 15–18]. Their diagnosis requires a multidisciplinary approach [19, 20] because a significant overlap of clinical and radiological features has been reported among these tumours. The knowledge of typical imaging features of IPMNs is crucial for making a correct diagnosis, excluding not only other pancreatic cystic lesions but also peripancreatic structures which could simulate pancreatic disease [21].

Indeed, the aim of this review is to describe the imaging features of IPMNs, emphasizing the most important signs involved in the management of these neoplasms.

2. Cross-Sectional Imaging Features

IPMNs can develop at any point in the pancreatic ductal system. According to their site of origin, they are distinguished into [9]

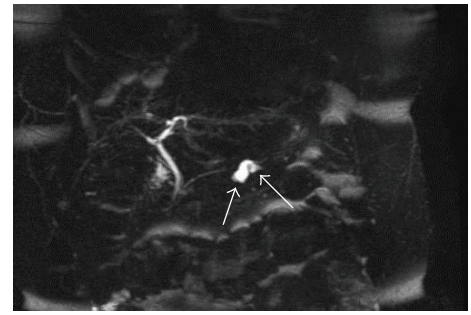


Figure 2: BD-IPMN in a 67-year-old female. MRCP acquisition clearly shows a cystic lesion centred on the body of pancreatic parenchyma (white arrows). The cyst shows a curved tubular shape. Due to the absence of high-risk-stigmata and worrisome features, lesions were safely managed.

(i) main duct IPMNs (MD-IPMNs)

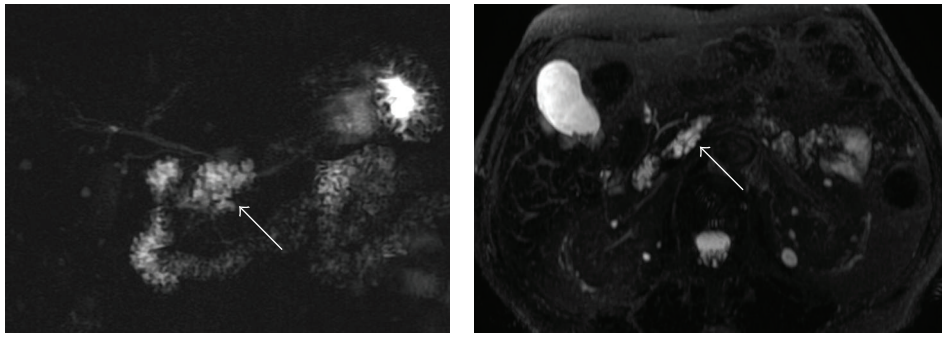
(ii) branch-duct IPMNs (BD-IPMNs)

(iii) both (mixed type).

Main radiological features of IPMNs have been reported in a popular pictorial essay by Procacci et al. in 1999 [9].

MD-IPMNs originate from the main pancreatic duct and are also indicated as *"Primary IPMNs"* (Figure 1). They may exhibit a diffuse or segmental involvement of main pancreatic duct. BD-IPMNs, which develop from secondary branches of main pancreatic duct, have been also reported as *"Secondary IPMNs"* (Figure 2).

Several studies have documented the different biological behaviour of primary and secondary IPMNs. The possibility of malignant degeneration is strongly dependent on the site of origin because MD-IPMNs show a risk of progression of 60–92%, whereas IPMNs arising from secondary branches have a lower value of degeneration, approximately 6–40% [22, 23].



(a) (b)

Figure 3: MRCP images (a and b), obtained using 2D FSE sequence and 3D FRFSE technique, respectively. BD-IPMN of about 3 centimeters located in the uncinate process of pancreas, with a typical microcystic appearance. No other worrisome features were found by EUS; the patient was successfully enrolled in a follow-up program.

Mixed type includes a combined pattern of presentation, with involvement of both main pancreatic duct and secondary branches.

A recent "*European experts consensus statement on cystic tumours of the pancreas*" [24] clearly suggests that the main role of CT/MR imaging is "*to reduce differential diagnoses when a cystic pancreatic lesion is revealed by ultrasonography*." Thus, MR and MRCP play an important role in the identification of the relationship between cystic lesions and pancreatic duct system. In case of connection, a diagnosis of IPMN could be suggested [24], whereas when connection is not identified, alternative diagnoses should include serous cystadenoma or mucinous cystadenoma. These cystic neoplasms are differentiated on the basis of their architecture: honeycombing and microcystic appearance are generally associated with serous lesions, whereas oligocystic/macrocystic appearance is frequently encountered in cases of mucinous cystadenomas [3, 16, 24, 25]. In addition, site of lesion and gender are important factors used for differential diagnosis [25].

Currently, cross-sectional imaging modalities have high accuracy in the diagnosis and assessment of loco-regional infiltration of cystic tumours of the pancreas; namely, CT has accuracy of 1.2–2.9%, whereas MRI reports higher values — 13.5–44.7% [24].

MRI and MRCP clearly distinguish the cystic dilatation of main pancreatic duct due to their high contrast resolution. Two-dimensional single shot fast spin echo (SSFSE) sequences and three-dimensional (3D) fast recovery fast spin echo (FRFSE) sequences are generally able to demonstrate the dilatation of main pancreatic duct or the cystic lesion originating from main duct (Figure 2). 3D FRFSE sequences may recognize the dilatation of main pancreatic duct also using multiplanar reconstruction (MPR) or maximum intensity projection (MIP) postprocessing techniques (Figure 3) [24, 26].

MPR images are strongly recommended for the identification of the communication of secondary IPMNs with main pancreatic duct. In a recent study by Sahani et al. [27] CT and MRCP were compared in the assessment of BD-IPMNs. For cyst communication, the overall sensitivity values of multidetector CT and MRCP were, respectively, 83% and 87%. Due to their high diagnostic performance, MPR/MIP postprocessing need to be performed simultaneously during CT and MR/MRCP examinations [24].

The goal of both cross-sectional imaging modalities—CT and MR with MRCP—is to identify some imaging features reported as "high-risk stigmata" or "worrisome feature" in the evaluation of pancreatic cysts. "High-risk stigmata" include essentially main pancreatic duct dilatation ≥10 mm (Figures 1 and 4) and the presence of solid components showing enhancement after contrast administration [28].

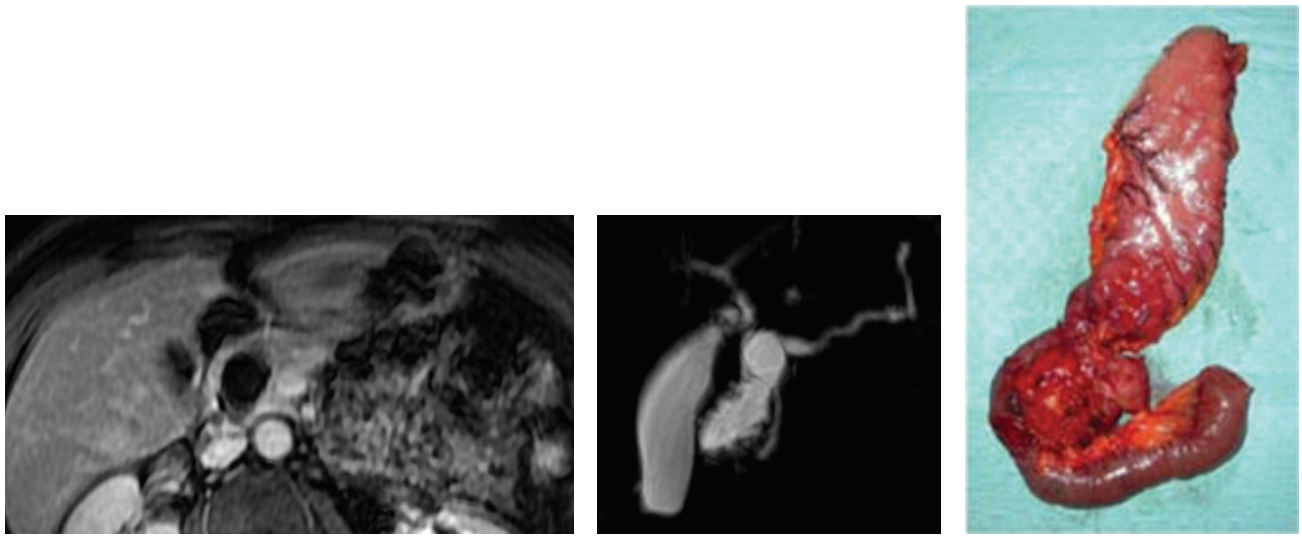
"Worrisome features," reported by IAP, are size of cyst ≥3 cm, thickened cyst wall with enhancement after contrast administration, mural nodules without enhancement after contrast, main duct with diameter of 5–9 mm, abrupt change in the main pancreatic duct caliber with distal pancreatic atrophy, and lymphadenopathy [28].

2.1. MD-IPMNs. MD-IPMNs are usually located in the proximal portion of the gland (75%), but they can also be recognized in the rest of the pancreatic parenchyma [29]. Main pancreatic duct dilatation is the typical radiological feature observed in primary IPMNs, involving the full length of the duct; segmental or diffuse dilatation of main pancreatic duct should exceed 5 mm, even if recent articles report that a lower size (5 mm) could be also adopted for the diagnosis of MD-IPMNs [28].

The measurement of main pancreatic duct is a crucial step in the evaluation of MD-IPMNs: a diameter of 5–9 mm is considered a "worrisome feature," whereas main duct measurement ≥10 mm is reported as "high-risk stigmata."

Both CT and MRI images could demonstrate the increased size of the duct, as its progressive dilatation could induce a parenchymal atrophy (Figure 1). Another typical finding observed in MD-IPMNs is the dilatation of the major papilla, the minor papilla, or both, with a bulging of the main pancreatic duct into the duodenal lumen [30]. Moreover, the diffuse main pancreatic duct dilatation is often associated with the dilatation of some branch ducts, particularly in the uncinate process and in the tail of the pancreas.

Both diffuse and segmental primary IPMNs have been associated with malignancy in the case of mural nodules or



(a) (b) (c)

Figure 4: Axial T1-weighted spoiled gradient echo after gadolinium administration (a). 3D FRFSE MRCP sequence obtained using MIP reconstruction (b). Surgical specimen (c), from poster EPOS C-2228 presented in [15]. (a) shows a homogeneous cystic lesion centered in the head of pancreas. No intralesion solid components were observed. In (b), MIP reconstruction was useful to better appreciate the cystic morphology of the lesion due to main pancreatic duct enlargement. Again, high-risk stigmata (main duct caliber >1 centimeter) suggested surgical management. A pancreatoduodenectomy was performed and final diagnosis deposed for borderline IPMN.

internal solid components [3–5, 22, 29]. The presence of solid components with enhancement after contrast administration has been reported as "high-risk stigmata" [28]. For this reason, CT and/or MRI examinations with contrast administration are recommended to better assess enhancement of internal nodules in primary IPMNs.

The diagnosis of IPMNs with a segmental involvement of the main pancreatic duct may be difficult because segmental dilatation rarely evolves into the cystic appearance (Figure 4). If the lesion is localized in the body or in the tail of the pancreas, the remainder of gland is normal. When lesion is located in the pancreatic head, it is often associated with upstream dilatation of the main pancreatic duct [9].

Primary IPMNs with cystic appearance require a differential diagnosis from mucinous cystadenoma. The dilatation of main pancreatic duct is generally observed in cystic IPMNs, whereas mucinous cystadenoma is rarely associated with main duct dilatation [9].

Primary IPMNs should be differentiated from chronic pancreatitis. Kim et al. investigated main radiological features which could be helpful for the differential diagnosis. These features include "duct dilatation without stricture, bulging ampulla, nodule in a duct, a grape-like cyst shape, and nodule in a cyst" [30].

The presence of internal nodules is more frequently associated with IPMNs than with pancreatitis. MRCP images clearly depict nodules and papillary projections, which usually appear as filling defects within the cystic lesions. However, in chronic pancreatitis ductal calcifications could simulate solid components, with hypointense signal on T2-weighted images. CT scan is able to demonstrate calcifications and help radiologists in the differential diagnosis between the two clinical entities. In addition, as reported by Kim et al., the presence of stone is considered one of the most specific signs of chronic pancreatitis [30].

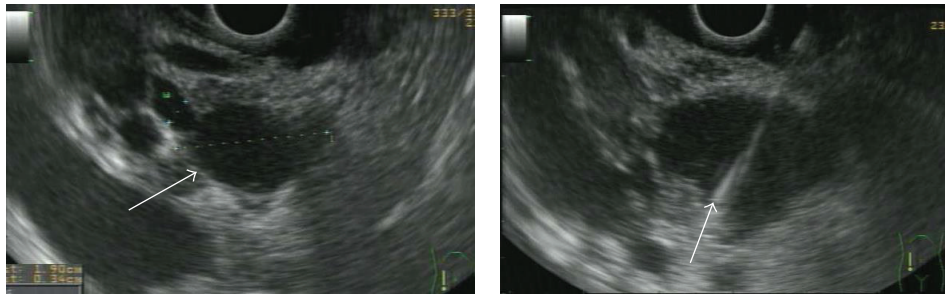
2.2. BD-IPMNs or "Secondary IPMNs". BD-IPMNs or *"secondary IPMNs"* (Figure 2) appear as cystic masses and therefore their demonstration is easier than MD-IPMNs. The most involved pancreatic region is the uncinate process (Figure 3). Lesions can be arranged in a microcystic or macrocystic pattern.

The microcystic pattern is characterized by small cystic lacunae separated by thin septa. This aspect is similar to that of serous cystadenoma and only the demonstration of a communication between the lesion and the main duct permits a correct diagnosis.

The macrocystic pattern is the most frequent. Lesions show a unilocular or multilocular architecture. The demonstration of the communication with the main pancreatic duct is a sign of differentiation from other cystic lesions such as the mucinous cystadenoma. However, the communication with main duct is often not appreciable on MR images [3, 22].

Thickness and irregularity of the tumor wall and of the septa are variable and increase with malignancy. Namely, increased thickness of cyst wall, showing enhancement after contrast administration, and/or mural nodules without contrast enhancement represent worrisome features that radiologists should always include in their report [28]. Other worrisome features that have to be considered are cyst size exceeding 3 cm and main pancreatic duct caliber of 5–9 mm [28].

Other imaging features have to be considered before making a differential diagnosis. Mucinous cystadenoma may



(a) (b)

Figure 5: Linear EUS image of a MD-IPMN (a): a lobulated anechoic cystic lesion is clearly depicted (white arrow). (b) shows EUS-FNA of the same lesions. In this lesion (about 3 cm in size), the absence of mural nodules and positive or suspicious cytology allowed a conservative management.

exhibit peripheral calcifications, which could reproduce an "eggshell" appearance [31]. Also, favourite locations in the pancreatic parenchyma are different for the lesions because secondary IPMNs are very often reported in the uncinate process [29], whereas mucinous cystadenoma is generally encountered in the body or in the tail of the pancreas.

IPMNs need to be differentiated from pancreatic pseudocysts, which develop as a complication of pancreatitis in up to 20–40% of cases [30]. In a recent work, "a grape-like appearance" has been associated with IPMNs in 79% of cases, whereas a unilocular cyst shape was reported in 34% of patients affected by chronic pancreatitis. However, unilocular secondary IPMNs are very difficult to differentiate from pseudocysts. Careful collection of clinical history is very important in these cases because pseudocysts generally develop as a complication of a severe episode of pancreatitis.

BD-IPMNs could be observed in a multifocal appearance. In this pattern of morphological presentation, IPMNs are divided into five classes: diffuse, proximal, proximally diffuse, distal, and bridge morphology [22]. The multifocality of IPMNs is responsible for an increased cumulative risk of neoplastic degeneration [32]. In this case, patients need to be followed over time in order to identify early signs of progression or degeneration.

2.3. EUS. Endoscopic ultrasonography (EUS) plays an important role in the diagnostic evaluation of IPMNs due to the possibility to collect fluid from cystic lesions. It can provide high resolution contrast images of pancreatic cystic lesions, demonstrating many important details about cystic lesions, such as wall thickness, presence of septa, and mural nodules [33]. In addition, it permits measurement of the pancreatic ducts and provides visualization of communication between cystic lesions and main pancreatic duct. Also strictures could be visualized along the course of main duct, contributing to the differential diagnosis between chronic pancreatitis and MD-IPMN [34–36].

In addition, EUS is able to guide fine-needle aspiration (FNA) (Figure 5) [37]. The fluid content could be analysed for the presence of oncological marker.

It has been well documented that CEA and CA 72.4 levels in the cystic fluid of the mucinous lesions are much higher (typically over 800 ng/mL) than those of nonmucinous ones [38]. Moreover, CEA and CA72.4 levels are higher in malignant mucinous neoplasms [39–43]. In a work by Brugge et al. a level of 192 ng/mL for CEA has a diagnostic sensitivity of 75%, a specificity of 84%, and an accuracy of 79% in differential diagnosis of mucinous and nonmucinous cysts [41].

In view of these considerations, several studies have recently investigated the diagnostic and prognostic values of these markers in order to establish the risk of malignant degeneration. Also inflammatory mediator proteins (cytokines, chemokines, and growth factors) — contained in pancreatic cyst fluid — could be used as potential diagnostic biomarkers able to characterize IPMNs [44]. However, sensitivity and specificity observed are not so high; detection of K-ras mutation in the pancreatic fluid can indicate the presence of a malignant cystic lesion, even with poor sensitivity (20%) [29]. The reported threshold level of 192 ng/mL for CEA has been evaluated as a predictor value of malignancy for IPMNs in a recent work by Kucera et al. [45]. The authors found that the mean level of intracystic CEA increases progressively from low-grade to high-grade of dysplasia (ranging from 1.261 ± 1.679 ng/mL to 10.807 ± 36.203 ng/mL). Among invasive cancers, the mean level reported was lower than IPMNs with various degrees of dysplasia. The reported sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of a cyst fluid CEA concentration greater than 200 ng/mL for the diagnosis of malignant IPMN—including lesions with high-grade dysplasia and invasive IPMNs—were, respectively, 52.4%, 42.3%, 42.3%, 52.4%, and 46.8% [45].

On the basis of the mentioned studies, EUS—even with FNA—does not show such high values of sensitivity and specificity in the diagnosis of IPMNs. In addition, it is an invasive [24], heavily operator-dependent modality that requires patient sedation [37]. Recent *"European expert consensus statement on cystic tumours of the pancreas"* remarked that EUS is *"an invasive diagnostic procedure,"* which needs to be performed after cross-sectional imaging (CT/MRI), in a multimodality imaging assessment of cystic pancreatic neoplasms [24].

After CT/MRI examinations, "All cysts with worrisome feature or cysts exceeding 3 cm in size without worrisome feature" should be investigated by EUS [28]; identification

of mural nodules, main duct signs of involvement by disease, or a cytology suspicion could suggest surgery [28].

Recently, some authors have proposed EUS imaging in the follow-up evaluation of secondary IPMNs. Kamata, in a recent retrospective study, compared the diagnostic value of EUS, ultrasonography, CT, and MRI in the assessment of pancreatic ductal adenocarcinoma arising from MD-IPMNs [46]. The population study included a total of 169 patients. All the mentioned imaging modalities followed 102 patients having side branch IPMNs without mural nodules and symptoms. The follow-up was performed in order to verify the incidence of IPMN-derived and/or concomitant pancreatic ductal adenocarcinoma. At the first follow-up examination, 17 IPMN-derived and 11 concomitant ductal adenocarcinomas were detected by the authors, with EUS overall sensitivity higher than other imaging modalities. For the entire follow-up period of the study, EUS maintained its better diagnostic accuracy in the detection of concomitant duct adenocarcinoma. Other authors have performed a follow-up study through US and MRCP in a large series of patients ($n = 109$) with BD-IPMNs [29]. In this study, EUS and ERCP were performed only in select cases, when the diagnosis was still unclear or doubtful after conventional cross-sectional imaging modalities.

However, the invasiveness and the variability represent limitations to adopting EUS in the follow-up of MD-IPMNs.

3. Management

Currently, management of IPMNs is one of the most debated topics in literature, and it is essentially based on cross-sectional imaging modalities (CT/MR) and EUS. There is no sufficient evidence for pancreatoscopy in management of cystic tumours and subsequently for IPMNs [24]. ERCP could be useful in selected cases, for example, in the evaluation of primary IPMNs with diffuse dilatation of main pancreatic duct, without evidence of mural nodules. In these cases, the diffuse increased caliber of main duct with bulging of major papilla promotes the right diagnosis of MD-IPMNs and could suggest the correct surgical approach.

First of all, cross-sectional imaging modalities should be able to clearly distinguish the three radiological patterns of presentation. As previously reported, primary IPMNs show a progression risk higher than secondary forms. In addition, multifocal branch-duct IPMNs have a cumulative risk of malignancy degeneration due to the coexistence of many cystic lesions.

High-risk stigmata, represented by dilatation of the main pancreatic duct equal to or more than 10 mm and/or solid components with enhancement after contrast, in view of its frequent association with malignancy, require surgical treatment. In fact, in a study performed by Abdeljawad, the prevalence of malignancy in 52 patients with pure main duct IPMN was analysed [47]. Among 16 asymptomatic patients reporting IPMNs, 4 had malignant lesions. In the symptomatic group (36 out of 52 patients), 25 lesions were malignant on histology. The size of the main pancreatic duct was analysed by authors using ROC analysis, and the largest area under the curve used to distinguish between benign and malignant MD-IPMN was found using a threshold level of main pancreatic duct of 8 mm (0.83; 95% CI = 0.72–0.94).

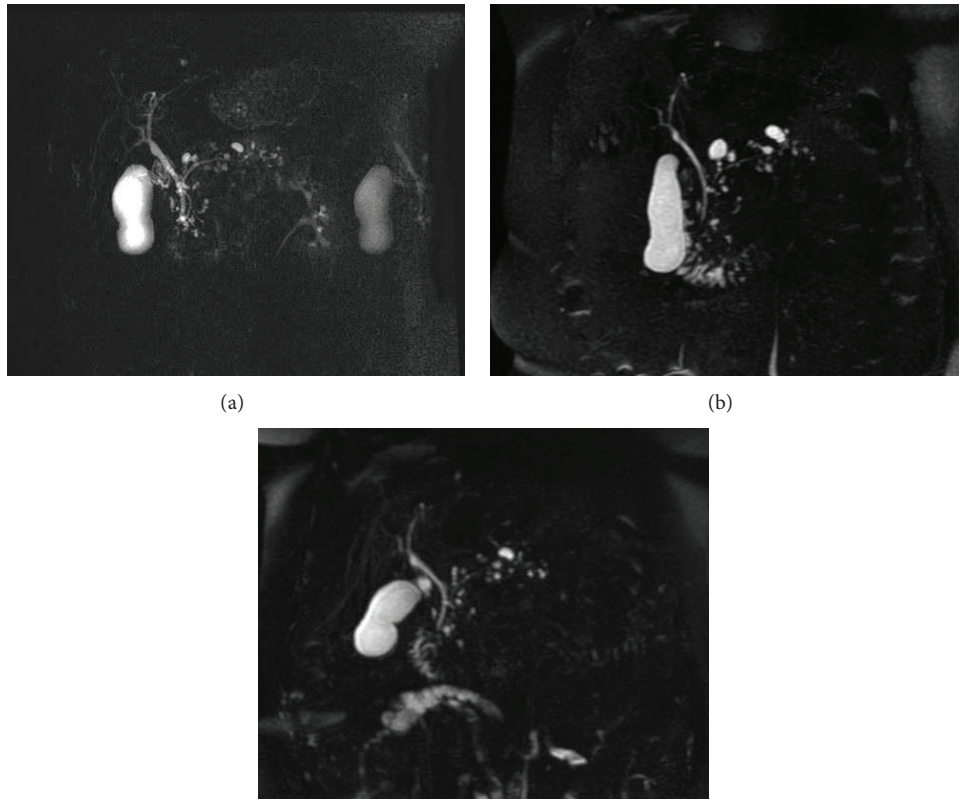
Worrisome features—including cyst size ≥3 cm, thickened cyst wall with enhancement after contrast administration, mural nodules without enhancement after contrast, main duct with diameter of 5–9 mm, and abrupt change in the main pancreatic duct caliber with distal pancreatic atrophy and lymphadenopathy—require further investigation [28]. As previously reported, EUS plays an important role in the management because confirmation of worrisome features could require a surgical treatment [28]. If absent, IPMNs could be monitored using MR/MRCP at 3 months and EUS annually for the first 2 years [28].

Regarding the size, cysts exceeding 3 cm, even if considered a worrisome feature, did not show a high value of correlation with malignancy. In a series observed by Sahani et al., only 5 out of 8 lesions with diameter >3 cm were malignant at pathological examination. In another series of 26 patients with secondary IPMNs reported by Manfredi et al., a significant change in the size of cystic lesions was observed. However, this imaging finding does not necessarily correlate with malignant transformation or increased suspicion of malignancy [4].

Therefore, the presence of nodules is probably the most significant change which needs to be carefully evaluated because it is strongly suspected as an indicator of malignancy.

Salvia has evaluated nonoperative management of secondary branches IPMNs in a prospective study, by performing contrast enhanced US and MRCP. Lesions were less than 3.5 cm in diameter and without nodules or solid components. Their study included a total of 109 patients. A first group (20 patients, 18.3%) required immediate surgery for the presence of symptoms or clinical and morphological features associated with malignancy. Among this group, the authors found only 2 patients with invasive carcinoma and 1 patient with carcinoma in situ. The remainder of the patients were evaluated with an average follow-up of 32 months. After an average follow-up of 18.2 months, Salvia et al. [29] reported only 5 patients with an increase in the size of the lesion. These patients underwent surgery and their final diagnosis was branch-duct adenoma in 3 cases and borderline lesions in 2 patients [29]. Thus, this study confirms that BD-IPMNs could be managed by imaging.

Finally, secondary IPMNs arranged in a multifocal pattern (Figure 6) should be evaluated for their increased risk of degeneration [48]. However, in another study, Salvia examined a total of 131 patients having multifocal secondary IPMNs. Here, only 10 patients were surgically managed, whereas the majority was followed for an average period of 40 months. 121 patients were conservatively managed, and they remained asymptomatic, without nodules or increase in diameter of their lesions. As reported by the authors, IPMNs in a multifocality setting could also be managed in a safe and reliable way [49].



(a) (b) (c)

Figure 6: Coronal MRCP acquisitions in an asymptomatic 70-year-old female patient with an incidental radiological finding of multiple pancreatic cystic lesions; MRCP exams were performed in 2009 (a), in 2012 (b), and in 2013 (c). Multiple small cystic lesions in the pancreatic parenchyma are clearly depicted in the three MRCP acquisitions, some of them showing a typical connection to the main pancreatic duct. This typical radiological pattern suggests the diagnosis of multifocal BD-IPMNs. No main pancreatic duct dilatation is observed. Cystic lesions do not show intraluminal solid components or mural nodules. Over time the MRI monitoring initially showed a mild enlargement of the lesions (from a to b) and then a size-reduction (from b to c). As reported in literature, IPMNs in a multifocal setting could also be managed in a safe and reliable mode.

4. Conclusion

Gastroenterologists, radiologists, and surgeons should be confident utilizing all imaging features of IPMNs.

On the basis of the diagnostic patterns analysed,

(i) radiologists should distinguish between primary, secondary, and mixed IPMNs; cross-sectional imaging features need to clearly demonstrate the relationships between IPMNs and pancreatic duct system;

(ii) identifying high-risk stigmata or worrisome features is recommended in order to suggest the correct management;

(iii) in case of IPMNs with high-risk stigmata, a surgical approach is needed, namely, for lesions with marked dilatation of the main pancreatic duct (≥1 cm) or showing internal solid enhancing components;

(iv) if worrisome features are depicted on cross-sectional imaging modalities, EUS investigation is required. Confirmation of these worrisome features requires surgery. In their absence, a follow-up procedure by CT/MRI could be safely adopted, monitoring the development of malignant signs.

Finally, all imaging features should be related to clinical conditions of patients (age, comorbidities, and performance status) for a correct management of the disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Hind Right Approach Pancreaticoduodenectomy: From Skill to Indications

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Background. Pancreaticoduodenectomy is the potentially curative treatment for malignant and several benign conditions of the pancreatic head and periampullary region. While performing pancreaticoduodenectomy, early neck division may be impossible or inadequate in case of hepatic artery anatomic variants, suspected involvement of the superior mesenteric vessels, intraductal papillary mucinous neoplasm, and pancreatic head bleeding pseudoaneurysm. Our work aims to highlight a particular hind right approach pancreaticoduodenectomy in selected indications and assess the preliminary results. *Methods*. We describe our early hind right approach to the retropancreatic vasculature during pancreaticoduodenectomy by mesopancreas dissection before any pancreatic or digestive transection. *Results*. We used this approach in 52 patients. Thirty-two had hepatic artery anatomic variant and 2 had bleeding pancreatic head pseudoaneurysm. The hepatic artery variant was preserved in all cases out of 2 in which arterial reconstruction was performed. In nine patients with intraductal papillary mucinous neoplasms the pancreaticoduodenectomy was extended to the body in 6 and totalized in 3 patients. Seven patients with adenocarcinoma involving the portomesenteric axis required venous resection and reconstruction. *Conclusions*. Early hind right approach is advocated in selected cases of pancreaticoduodenectomy to improve locoregional vascular control and determine, safely and early, whether there is mesopancreas involvement.

1. Introduction

Pancreaticoduodenectomy (PD) is the treatment of choice for malignant and several benign conditions of the pancreatic head and periampullary region [1–4]. Since the first PD performed by Whipple in 1937, more than 70 technical improvements have been made, mainly related to pylorus preservation or reconstruction of pancreatico-digestive continuity and much less regarding the type of resection [1, 5–7]. Standard PD is usually performed with transection of the pancreatic neck before the superior mesenteric artery (SMA) dissection [8–10]. However, since the limited involvement of portomesenteric vein is no longer considered unresectable disease, the resectability is now assessed by whether or not the SMA is involved [11]. Moreover, the extended indications of PD in case of tumors associating hepatic artery (HA) variants or invading the mesentericoportal axis (borderline resectable pancreatic head adenocarcinomas) [11], as well as the importance to achieve R_0 posteromedial resection margins (in adenocarcinomas and main duct-intraductal papillary mucinous neoplasms-MD-IPMN) [12], led to the development of so called "artery first" approaches [13]. Notable amongst these is an early right posterior approach to the superior mesenteric vessels, with mesopancreas (MP) dissection close to the origin of the SMA. The aim is to assess the resectability before taking an irreversible step, and variants of the arterial blood supply to the liver, to undertake the mobilization of the specimen before pancreatic or digestive division, and, if necessary, the safe venous clamping [5, 13–26].

We have adopted this hind right approach to the SMA since 2007 and have been using it combined with the early isolation and dissection free of the superior mesenteric vein (SMV) beneath the pancreas, as our "standard approach" PD in selected indications such as HA anatomic variants, suspected involvement of mesentericoportal axis or SMA, MD-IPMN, and pancreatic head bleeding pseudoaneurysm. It is very suitable to early assess the infiltration of SMV and SMA, allowing appropriate handling at the initial stages of the resection itself.

Although we have previously reported this approach related to PD in case of HA variants [5, 6], whereas we extended the indications, we describe how, when, and why to perform this modified right retropancreatic vascular approach PD and our current experience.

2. Methods

One hundred fifty consecutive patients have been registered for PD for benign and malignant diseases of the periampullary and pancreatic head region between January 1, 2007 and February 28, 2014. Among them, 52 (30 males and 22 females, median age 56.7 years; range 40–78 years) underwent PD with early hind right dissection. Patient characteristics are presented in Table 1. In 32 patients, the preoperative multidetector computed tomography revealed HA anatomic variants (Table 1): aberrant right HA (RHA) and replaced common HA (RCHA), with retropancreatic (28 cases) or intrapancreatic (4 cases) and retroportal course. Seven patients with adenocarcinoma had the added involvement of the portomesenteric vein. Nine patients with MD-IPMT were preoperatively assessed by abdominal multidetector computed tomography and endoscopic ultrasound with guided fine needle aspiration biopsy. A bleeding pancreatic head pseudoaneurysm was disclosed by computed tomography in 2 patients.

The procedures were performed by the same trained surgical team.

Table 1: Patient characteristics.

Characteristics	Number	%
Patients	52	
Median age (ys)	56.7	
Males/females	30/22	
ASA classification		
I	12	23%
II	33	63%
III/IV	7	14%
Diseases (pathologic entity)		
Malignant disease		
Total	**35**	**68%**
Pancreatic ADK	23	44%
Ampullary ADK	3	6%
Distal CBP ADK	6	12%
Duodenal ADK	2	4%
Neuroendocrine pancreatic tumours	1	2%
Benign Disease		
Total	**8**	**16%**
Insulinoma	3	6%
Chronic pancreatitis	5	10%
IMPT	**9**	**18%**
Hepatic artery anatomic variant		
Total	**32**	**61%**
(1) Aberrant right hepatic artery (RHA)	**24**	**47%**
Origin		
From the SMA	21	40%
From the CT	3	6%
Type		
Replaced RHA	17	32%
Accessory RHA	7	13%
(2) Aberrant common hepatic Artery (CHA)	**8**	16%
Origin		
From the SMA	7	13%
From the aorta	1	2%
Type		
Replaced CHA	8	16%

3. Surgical Technique

The pancreas head is exposed by an extended Kocher maneuver carried out beyond the aorta, incision of the attachment of the transverse mesocolon to the right Gerota fascia, and opening of the lesser sac by separating greater omentum and transverse colon using a Liga-Sure device. The superior mesenteric vein (SMV) is early isolated below the pancreas, where it passes over the third duodenum and is dissected free from the pancreas and uncinate process (Figures 1 and 2), with ligation of the right gastroepiploic and inferior pancreaticoduodenal veins and early creation of a tunnel between pancreas and portomesenteric axis towards the hepatic pedicle (Figures 2 and 3). This step early detects whether or not the portomesenteric vein is involved and assesses the infiltration status of the infrapancreatic SMA. Behind the pancreas, the dissection must surpass the aorta to get full posterior leftwards mobilization of the duodenopancreas, and the plane between the SMV and the SMA is identified as part of the MP (Figures 2 and 3).

The retropancreatic dissection is carried on downwards from the inferior border of the Winslow foramen along the Treitz fascia, exposing the inferior vena cava on its left side, the upper margin of the left renal vein, and, in between, the origin of the SMA along with the posterior pancreatic capsule (Figures 2, 3, and 4).

The SMA origin is identified in this angle, and along its adventitial plane the MP (including RPL or retropancreatic medial margin) is dissected and removed "step-by-step." The MP is inserted on its right aspect, in a frontal plane behind the pancreas (Figures 2, 3, and 4). This dissection

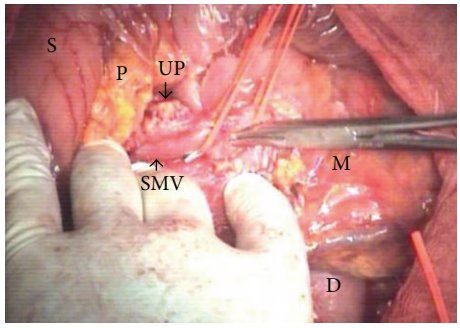

Figure 1: Early exposure of the SMV beneath the pancreas, on the anterior third duodenum; the vein is dissected free from the pancreas and uncinate process. SMV: superior mesenteric vein; D: duodenum; M: mesentery; P: pancreas; S: stomach; UP: uncinate process.

is pursued over 3-4 cm, from the SMA origin until its entrance into the mesentery, using progressive exposure and gentle medial retraction of the portal vein (PV), which is also freed from the MP (Figures 3 and 4). The superior and inferior pancreaticoduodenal arteries are identified and ligated (Figure 4). The MP is retracted to the right and all lymphatic and perineural tissue between SMA and SMV is removed to achieve negative resection margins. Possible SMA invasion can be early detected, due to MP involvement, avoiding the risk of nonradical resection. Complete excision of the connective tissue between the origin of the SMA and the (CT) is performed as well. This exposure enables the dissection of a RHA originating from SMA or CT (Figure 3) or a RCHA arising from the SMA (Figures 4 and 5). The vessel usually arising 1-2 cm from the SMA origin is looped and freed from the MP, upwards to the hepatic pedicle (Figures 3, 4, and 5). Its safeguarding is generally possible. To facilitate the SMA and aberrant RHA or RCHA dissection, the duodenopancreas is retracted en bloc upwards, ventrally and to the left. Limited dissection along the right side of the SMA is advocated (Figures 2, 3, and 4), to avoid extensive removal of the perivascular nervous plexus, resulting in postoperative intestinal motility troubles.

The hepatic pedicle is approached after this extended dissection. The cholecystectomy is performed, the common and proper HAs are isolated, and the right gastric vessels and gastroduodenal artery are identified and clamped to make sure that the arterial flow either in hepatic or gastric arteries remains normal and there is no unrecognized CT stenosis. The gastroduodenal artery is divided, as well as the common bile duct above the entry of the cystic duct (Figures 3 and 4). This improves the exposure of the suprapancreatic PV. During periportal lymphadenectomy one should be aware of an eventual accessory or replaced RHA originating from the SMA or CT, or to a RCHA from the SMA. If present, this vessel running upwards behind the PV is looped (Figures 3 and 4). The SMV is entirely dissected at the inferior pancreatic margin with ligation of all veins draining the uncinate process, which is exposed up to the right side of the SMA. At this stage the posterior wall of the portomesenteric axis is entirely exposed. The Treitz ligament is divided, allowing mobilization of the duodenojejunal junction, so the specimen to be removed reaches the right side of the mesenteric root.

Once the radicality of PDR is established jejunal and distal gastric division are undertaken according to Whipple procedure using stapling devices. The last step of the resection is the pancreatic neck transection, just in front of the PV using a usual "cold" scalpel. When pancreatic division must be deviated towards the body, the dorsal pancreatic artery and collaterals of both the SMA and the SMV (from the lower edge of the pancreas) are divided. In case of involvement of the portomesenteric confluence, the splenic vein is controlled behind the body. Adequate mobilization of the mesentery and right colon is necessary to perform safely "en bloc" resection and venous reconstruction. This mobilization is useful in case of limited portomesenteric invasion, in order to avoid vein grafting during venous reconstruction.

In case of IPMN extending from the head to the body, the retropancreatic mobilization is done leftwards and the splenic vessels are dissected with successive ligation of their collaterals. When the pancreatic body is mobilized sufficiently the pancreas can be divided at any level, or entirely removed. Frozen section analysis is performed at the sectioned champs, to assess the malignant status of the remnant pancreas. Reconstruction phase, drainage, and postoperative care are similar to those from standard PD. During the operative procedure we use standard dissection and ligatures, monopolar section and coagulation. The LigaSure device is used during Kocher maneuver and division of the lesser and greater omentum.

4. Results

HA variants were intraoperatively confirmed in all 32 cases. The aberrant vessel was preserved in 30 cases. A RCHA originating from the SMA was involved by an enlarged lymph nodes mass behind the pancreatic head (borderline resectable pancreatic head cancer) in 2 patients, so a segmental resection of the involved RCHA had to be performed with arterial reconstruction, using the reversed splenic artery in both cases. Right hind approach PD was also performed in emergency in two cases of pancreatic head bleeding pseudoaneurysm, with early ligation of the pseudoaneurysm feeding artery, originating from the inferior pancreaticoduodenal artery. Seven patients with borderline resectable ductal adenocarcinoma involving the portomesenteric confluence required en bloc resection, mobilization of the right colon, and mesentery root followed by mesentericoportal venovenous suture. When vascular reconstruction was required, clamping time did not exceed 22 minutes. Anastomotic patency and normal blood flow were confirmed by Doppler ultrasound at the end of the procedure.

The same approach was used in 9 patients with MD-IPMN (6 PD extended to the body-IPMN in the head, uncinate, or neck and 3 total PD-IPMN diffusely involving the main pancreatic duct).

Since we routinely perform this approach in selected indications, no conversion to standard PD was undertaken.

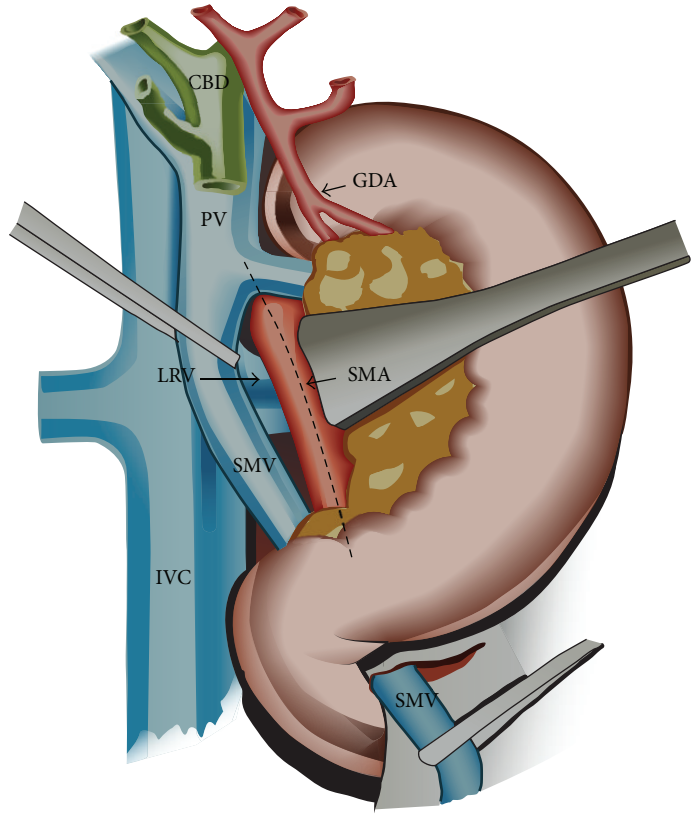

Figure 2: Hind right approach PD: after extended Kocher maneuver the duodenopancreas is retracted upwards and medially. Early posterior approach to the SMA (dissection of the MP along the discontinuous line) combined with an early approach to the SMV below the pancreas, where it passes over the third duodenum (the SMV is dissected free from the pancreas and uncinate, the right gastroepiploic and inferior pancreaticoduodenal veins are ligated, and a tunnel is created between the portomesenteric vein and pancreas towards the hepatic pedicle). CBD: common bile duct; GDA: gastroduodenal artery; IVC: inferior vena cava; LRV: left renal vein; P: pancreas; PV: portal vein; SMA: superior mesenteric artery; SMV: superior mesenteric vein.

The median operative time was 295 minutes (range 225–435) and median blood loss was 760 mL (range 215–1090). The short-term outcome related to this approach is shown in Table 2. For the malignant tumors, a R_0 resection was achieved in 32 patients and a R_1 resection in 5 patients (14%), (all with borderline resectable pancreatic head cancers). No R_2 resection was noted. The follow-up has lasted until patient death or until the cut-off date of February 28, 2014. The median follow-up time was 32.5 months (range 6.5–72). At the time of the last follow-up, 39 patients were still alive. If only patients with pancreatic cancer were taken into account, median survival time was 19.1 months (range 8.5–32).

Table 2: Short-term outcome after pancreaticoduodenectomy with early hind right dissection.

Surgical complications (27 events in 22 patients) (42%)	
Pancreaticojejunostomy leak	7 (13%)
Remnant pancreas acute pancreatitis	2 (4%)
Delayed gastric emptying	9 (17%)
Pancreatic stump hemorrhage	2 (4%)
Hemorrhage from gastric stapled suture	2 (2%)
Wound infection	5 (10%)
Relaparotomy	3 (6%)
Hospital mortality	2 (4%)
Median hospital stay (days)	16

5. Discussion

Because of continuous decrease in mortality rate, PD is nowadays routinely performed for tumors of the pancreatic head and periampullary region, with or without invasion of the mesentericoportal axis and even in IPMN. The early hind approach to the MP during PD on the right side of the SMA, before the digestive and pancreatic continuity that should be interrupted, is of particular interest in case of HA abnormality, with RHA originating from SMA or CT, or RCHA from the SMA, suspected involvement of the SMA, MD-IPMN extended from the pancreatic head to the body, and involvement of the portomesenteric axis. Recently we performed in emergency this hind right approach Whipple in

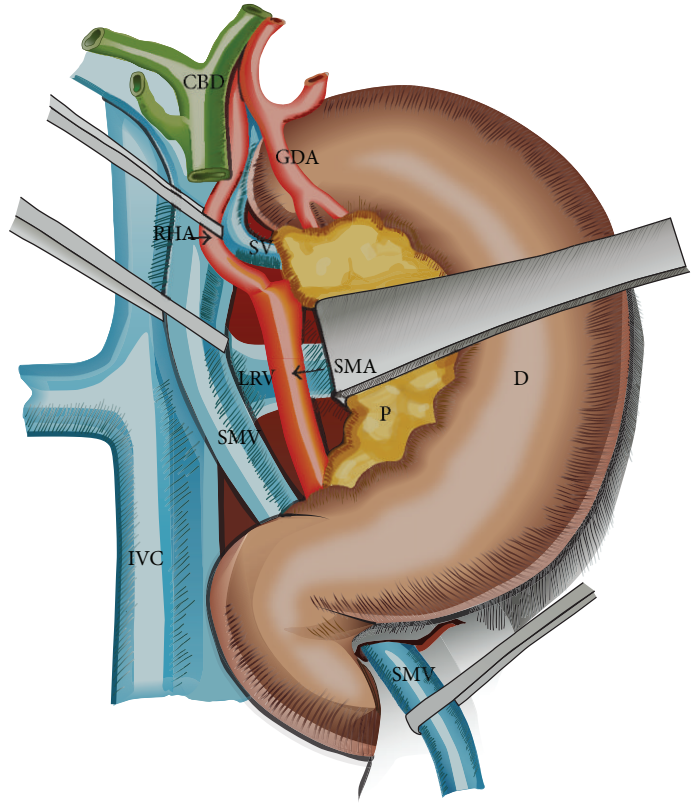

Figure 3: Hind right approach PD. Within the MP dissection, the SMA origin is detected in the angle between the left border of the IVC and the upper margin of the LRV. A RHA arising from the SMA is easily isolated 1-2 cm beneath SMA origin. The aberrant vessel is dissected upwards to the hepatic artery and preserved. The SMA is dissected along 4 cm towards the mesenteric root. CBD: common bile duct; D: duodenum; GDA: gastroduodenal artery; IVC: inferior vena cava; LRV: left renal vein; P: pancreas; SV: splenic vein; SMA: superior mesenteric artery; SMV: superior mesenteric vein.

two patients with bleeding pancreatic head pseudoaneurysm. In this setting, early ligation of the inferior pancreaticoduodenal artery (feeding the bleeding pancreatic head pseudoaneurysm) enabled a steady hemostasis and removal of the lesion. From the technical variants described as "artery first" approach to PD [13], we routinely adopted a right posterior approach to the SMA, additionally combined with early isolation and dissection free of the SMV beneath the pancreas (Figures 1, 2, and 3). It has become the standard practice in our unit in the above mentioned indications. The potential advantage of this approach is that technical difficulties, which may be encountered either due to tumor infiltration of the SMA, SMV, or the main PV, can be clearly assessed and handled appropriately at the initial stages of the resection itself. It gathers the advantages of a posterior "artery first" approach PD with a modified uncinate process first approach, regarding the following:

(1) identification of SMA involvement either at the origin or at uncinate;

(2) identification of the portomesenteric vein involvement requiring en bloc resection;

(3) identification and preservation of HA variants;

(4) adequate retropancreatic lymphadenectomy;

(5) minimal bleeding by early ligation of the IPDA and IPDV;

(6) effectiveness in obesity, postchemotherapy status, and peripancreatic inflammation;

(7) mobilization of the whole gland before transection;

(8) removal of large tumors of the pancreatic head extending to the uncinate.

Standard PD implies the creation of a tunnel between the pancreatic neck and the PV, followed by neck transection so the pancreatic continuity is interrupted before radicality of the resection can be assessed. The late determination of the MP infiltration status means that the surgeon is already committed to resection. Even in some recent series, nonradical PD is presented [27, 28]. Moreover, in the standard PD, dissection of a RHA or RCHA is usually performed late, when bleeding from the resection specimen decreases the exposure of the SMA and of an aberrant RHA. Early neck transection is also not suitable when the pancreatic neck and/or the portomesenteric axis are involved [18, 19] or in MD-IPMN extended to the body or diffusely affecting the pancreas [12, 16, 17]. One of the difficulties of PD lies in the variability of peripancreatic vascular anatomy. Preoperative assessment of variant pattern of the arterial

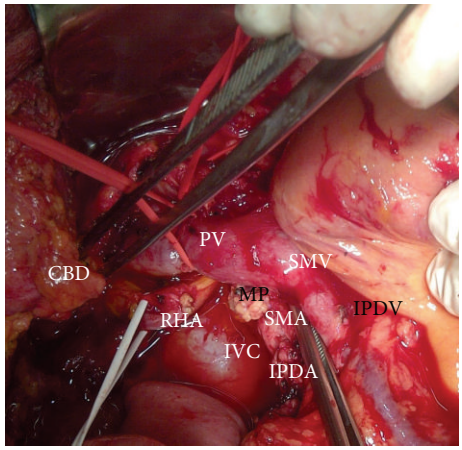

Figure 4: Hind right exposure of the MP and superior mesenteric vessels; the duodenopancreas with the tumor is retracted upwards and to the left; CBD: transected common bile duct; IVC: inferior vena cava; MP: dissected mesopancreas, between SMA origin and celiac trunk; PV: portal vein; RHA: right hepatic artery arising from the celiac trunk and retroportal path; SMA: superior mesenteric artery; SMV: superior mesenteric vein; IPDV: ligated inferior pancreaticoduodenal vein; IPDA: ligated inferior pancreaticoduodenal artery.

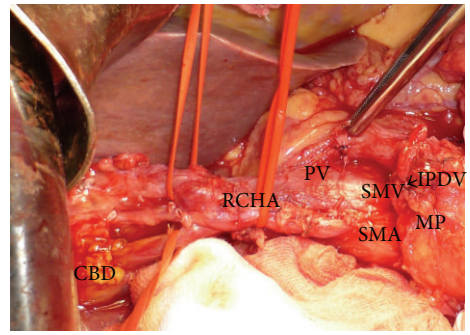

Figure 5: Hind right dissection of the SMA with resected MP and spared intrapancreatic path RCHA, just before the digestive and pancreatic transection; CBD: common bile duct; MP: mesopancreas; PV: portal vein; RCHA: replaced common hepatic artery with intrapancreatic path and early bifurcation in right and left hepatic branches; SMA: superior mesenteric artery; SMV: superior mesenteric vein; IPDV: ligated inferior pancreaticoduodenal vein.

blood supply to the liver (variants, strictures) is necessary to avoid unnecessary complications, such as fatal hepatic injury [29, 30]. Accidental ligation of HA may result in hepatic necrosis, ischemic biliary tract injury, or anastomotic complications [31, 32]. Moreover, injury of an aberrant HA during PD relates to a breakdown of bilioenteric anastomosis, because the blood supply to the cranial part of the common bile duct is entirely dependent on the RHA after PD [32–34]. RHA or RCHA from SMA may be situated behind or within the pancreas head or along its ventral side [35–37]. We could not confirm its course before dissecting and isolating it from the SMA origin within the MP dissection. In our series, in the vast majority of patients with HA anatomic variant, the aberrant vessel was spared. In two patients this artery was willingly sacrificed for oncological reasons, but reconstructed.

Pancreatic head carcinoma with venous limited involvement can be safely resected with a long-term survival similar to that observed after radical resection without venous involvement [13, 18, 23, 38, 39]. In such situation, the best option is to perform "en bloc" venous resection in order to obtain R_0 resection [11]. By using the hind right approach, after transection of the pancreatic isthmus, the tumor remains attached only to the involved veins, so clamping of the portomesenteric confluence is easier and shorter [39, 40]. Mobilization of the right colon and mesentery root are useful to avoid vein grafting during reconstruction of the PV [41]. Since the pancreatic transection is performed at the end, congestion and bleeding are less likely whereas the venous drainage of both the specimen and bowel are compromised minimally during most of the procedure [20, 25]. Moreover, there is a reduced intraoperative blood loss, due to an early ligation of the inferior pancreaticoduodenal artery [20, 40].

In MD-IPMN, the most frequent localization is the pancreatic head, but involvement of the body may occur [12, 16, 17]. In this setting and particularly in malignancies [12], en bloc resection requires pancreatic division of the body rather than the neck. Early hind right approach to the MP facilitates pancreatic mobilization towards the left. By the early approach to the SMV beneath the pancreas, our technique enables the total pancreatectomy as mobilization can be achieved without transecting gland. In fact, final transection of the pancreas can be performed at the desired place if it is enough separated from the splenic vessels, preventing tumor opening, which might disseminate cancer into the abdomen. Furthermore, dissection along the splenic vessels can be extended up to the splenic hilum allowing splenic preservation if the whole pancreas must be resected [12, 16, 17].

Removal of all small vessels, nerves, and lymphatic nodes and networks within the retroperitoneal adipose tissue, the so-called "Total Mesopancreas Excision," increases the rate of negative resection margins, thus reducing the local recurrence rate and improving the survival [20, 21, 42]. The MP is the retroperitoneal thin soft tissue, within retropancreatic attachments comprising connective tissue, perivascular nervous plexus, and lymph nodes belonging to posterior pancreaticoduodenal vessels. The term MP seems to replace or rather include the classical term of retroportal lamina (RPL), retroperitoneal/posteromedial margin, or retropancreatic capsule, which has a frontal disposition between the pancreas and the SMA [10, 14, 15, 20, 21]. The MP is of great interest with respect to curative resection in malignancies since it is the primary site for R_1 resection [21, 43]. In our series, the positive resection margins-R_1 (5 patients) were associated to borderline resectable pancreatic head cancers and extended to uncinate, but the rate (14%) is lower than that reported in the literature for similar cases (18–24%) [11, 44]. MP dissection remains one of the most challenging steps in PD no matter what type of approach is used (standard, posterior, or artery first below the pancreas). At present, there

is no evidence based on large series concerning the benefits of the "MP first" or "artery first" approaches over the standard PD [45]. A drawback of our study is the heterogeneity of the indications and pathology conditions for PD (malignant/benign diseases, IPMN). Therefore, a comparative study with a matching cohort of patients undergoing standard PD should be difficult. As a matter of fact, this was not an endpoint of our study, but to highlight the advantages of our approach in selected indications of PD. Further prospective randomized studies are necessary to assess the real clinical impact of the MP excision in achieving negative resection margins, decreasing local recurrence, and improving the long-term survival of patients resected for pancreatic cancer.

It is worth noting that a limitation of the retropancreatic approach PD was reported in obese patients and those with extensive peripancreatic inflammation [26]. Nevertheless, the surgeon should face the same concern in standard PD too. We encountered this problem in our patients with chronic pancreatitis and those with previous chemotherapy. These conditions render the procedure more difficult rather during vascular isolation and dissection. However, since our approach early exposes the retropancreatic vasculature and dissects the portomesenteric vein below the pancreas and uncinate, it comes to be useful even in peripancreatic inflammation, as it facilitates retropancreatic tunneling above PV and whole pancreas mobilization before transection.

In conclusion, early hind right dissection combined with early exposure of the SMV below the pancreas is a useful technique to expose the retro- and infrapancreatic mesenteric vasculature early during PD. Because of its advantages, we use it routinely in patients with HA anatomic variants, suspected SMA involvement, limited invasion of the mesentericoportal axis, MD-IPMN, and bleeding pancreatic head pseudoaneurysm. MP first dissection facilitates the radicality and safety of PD and enables early vascular control. Further prospective studies are required to assess its advantages over standard PD, since there is no consensus worldwide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Difference in Early Activation of NF-κB and MCP-1 in Acinar-Cell-Rich versus Fibrotic Human Pancreas Exposed to Surgical Trauma and Hypoxia

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Objectives. Previously we have shown that a pancreas with over 40% acinar cells is exposed to postoperative pancreatitis and other complications after pancreaticoduodenectomy (PD). Our aim was to analyze the expression of NF-κB and MCP-1 in the cut edge of human pancreas after PD in both acinar-cell-rich and fibrotic pancreata. *Methods.* Several pancreatic samples from six patients, three with acinar-cell-rich and three with fibrotic pancreata, were exposed to surgical trauma in PD, and thereafter to hypoxemia for 15 minutes, 2–2.5 hours, 4 hours, or 6 hours, to mimic postoperative conditions of the pancreatic remnant in a patient. Immunohistochemical analysis of inflammation markers (NF-κB, MCP-1) was performed. *Results.* In the acinar-cell-rich pancreata, intra-acinar NF-κB and MCP-1 expression increased from mild at 15 minutes to high during the first 4 hours, whereas in ductal cells MCP-1 staining was highly intense at both time points. Acinar cell NF-κB and MCP-1 expression and ductal cell MCP-1 expression were also observed in the fibrotic pancreata, but the activation remained low throughout the 6 hours. *Conclusions.* In acinar-cell-rich pancreas, an extensive inflammatory cascade begins almost immediately after surgical trauma. Fibrosis may limit the progression of inflammatory process in pancreas.

1. Introduction

Pancreaticoduodenectomy (PD) has become a standard operation with low mortality. However, at 42%–60% perioperative morbidity remains substantial. The most common complications include delayed gastric emptying, postoperative pancreatic fistula, wound infections, and postpancreatectomy hemorrhage [1–4].

A pancreas at high risk of severe complications can be predicted perioperatively. Acinar-cell-rich pancreas (defined as showing over 40% of acinar cells in the pancreatic transection line) is accompanied by an increased risk of postoperative pancreatitis or milder pancreatic irritation [3, 5, 6]. In our previous study [6], 92% of the patients with acinar-cell-rich pancreas developed postoperative complications. The complication rate decreased to 21% when there was more than 60% of fibrosis in the pancreatic transection line. We hypothesized that intraoperative pancreatic injury may immediately activate the inflammatory cascade in the remaining pancreas and that this activation may differ in acinar-cell-rich and fibrotic pancreas.

According to the prevailing theory, acute pancreatitis is set off by uncontrollable activation of trypsin leading to excitation of other digestive enzymes and, eventually, autodigestion and inflammation [7]. The inflammatory cascade, especially the signaling molecules involved, has been under intense scrutiny in recent years. Several signaling molecules have been shown to play important roles in the progression of the inflammation process in the pancreas. They include among others nuclear factor κB (NF-κB); monocyte chemoattractant protein 1 (MCP-1); interleukins IL-1, IL-2, and IL-6; platelet-activating factor (PAF); substance P; and tumor necrosis factor α (TNF-α) [8–10]. Both NF-κB and MCP-1 are shown to upregulate early in acute pancreatitis [9–13].

In animal models, NF-κB activates within 30 minutes and MCP-1 within 60 minutes in acinar cells after the induction of inflammation [11, 14] and this activation leads to exacerbation of acute pancreatitis [9, 15, 16].

The inflammatory cascade in human pancreas after surgical trauma has not been previously investigated. The aim of this study was to investigate postoperative inflammation in acinar-cell-rich and fibrotic human pancreas exposed to surgical trauma and hypoxia.

2. Materials and Methods

From among the patients undergoing PD in Tampere University Hospital, six individuals were chosen for the study based on the histopathology of the cut edge of the pancreas: three with acinar-cell-rich pancreas (>40% acini on the cut edge) and three with fibrotic pancreas (>60% fibrosis on the cut edge). In the acinar-cell-rich group, the patients and the final histopathological diagnoses were as follows: 50-year-old female with neuroendocrine carcinoma of the head of the pancreas, 55- and 57-year-old males with adenocarcinomas of the head of the pancreas. In the fibrotic group, the diagnoses were as follows: 78-year-old male with serous cystadenoma of the head of the pancreas, 60- and 74-year-old males with adenocarcinomas of the head of the pancreas.

During the operation, at the time of the transection, a tissue sample (size 2 mm thick, 10 mm in diameter) was harvested from the cut edge. The specimen was cut into five pieces which were immersed in physiologic NaCl solution to prevent them from drying. The tissue was thus exposed to surgical trauma followed by ischemia ex vivo, in an endeavor to mimic the conditions at the cut edge of the pancreatic remnant in the patient. At 15 minutes, 2–2.5 hours, 4 hours, or 6 hours, the NaCl solution was replaced by 4% paraformaldehyde and the samples were allowed to fix overnight. The samples were then dehydrated and embedded in paraffin. Sections (5 μm thick) were cut for immunohistochemical analysis.

Immunohistochemical analysis was performed using the following antibodies at the dilutions indicated: anti-NF-κB p50 (1 : 200; AbD Serotec, Oxford, UK) and anti-MCP-1 (1 : 200; AbD Serotec). Controls included omission of the primary antibodies and the use of nonimmunized mouse and rabbit IgG. The staining was performed with a broad-spectrum Histostain-Plus kit (Invitrogen, Camarillo, CA, USA) as previously described [17]. The sections were lightly counterstained with hematoxylin.

The slides were then subjected to microscopic analysis (Nikon Microphot-FXA). Quantitative analysis of NF-κB 15-minute and 4-hour samples was performed by two independent researchers (ML, MB). The percentage of activated acinar cells (stained nucleus) out of the total number of acini in each sample was determined from representative areas using a magnification of 250. The means (±SEM) of the three acinar-cell-rich and the three fibrotic samples were then calculated. Differences in the intensity of MCP-1 staining were determined semiquantitatively and expressed as low, moderate, or high.

The study protocol was approved by the ethics committee of Tampere University Hospital.

3. Results

NF-κB staining was seen in the nuclei of acinar cells, and MCP-1 activation was found in the cytoplasms of acini and ductal cells. Qualitative analysis revealed the progression of NF-κB activation in acinar-cell-rich pancreata during the 6-hour period (Figure 1) such that the highest NF-κB expression was at 4 hours (Figures 1 and 2). In the fibrotic pancreata, acinar cell activation of NF-κB was also detected, but the tissue expression of NF-κB did not increase over time (Figure 2). NF-κB-positive fibroblasts were scarce, with the fibroblast nuclei being predominantly unstained. In all tissue sections the intensity of NF-κB staining appeared even and no gradient from outside to inside was detectable.

Quantitative analysis for the acinar-cell-rich pancreata showed that acinar cell NF-κB activation increased from mild at 15 minutes (35% ± 7%, mean ± SEM) to high (74% ± 4%) during the first 4 hours (Figure 3). NF-κB activation was 30% (±6%) at 15 minutes and 35% (±4%) at 4 hours in the fibrotic pancreata (Figure 3).

Acinar cell expression of MCP-1 increased from low at 15 minutes to moderate during the first 4 hours in the acinar-cell-rich pancreata, whereas in ductal cells MCP-1 staining was highly intense at both time points (Figure 4). Acini and ductal cells did not express MCP-1 at 15 minutes in the fibrotic pancreata and only minor staining was observed at 4 hours (Figure 4).

4. Discussion

An acinar-cell-rich pancreas is at higher risk of post-PD complications than is a fibrotic pancreas. Intraoperative pancreatic injury may activate the inflammatory cascade differently in acinar-cell-rich pancreas and fibrotic pancreas. The role of inflammation markers in human pancreas following surgical trauma has not been previously studied and was the focus of this study. It was concluded that the intra-acinar cell inflammatory cascade may lead to pancreatitis almost immediately after induction of injury by surgical trauma and ischemia in acinar-cell-rich human pancreas, whereas fibrosis may limit the progression of inflammation in pancreas.

Several signaling molecules (such as IL-1, IL-2, IL-6, PAF, substance P, TNF-α, MCP-1, and NF-κB) have been shown to play important roles in the progression of experimental acute pancreatitis [8–10]. Studies have shown that both NF-κB and MCP-1 upregulate early in acute pancreatitis and may exacerbate its severity [9–16, 18, 19], which is why these markers were chosen for our study.

NF-κB has been shown to regulate the transcription of several genes involved in immunity and inflammation [9]. Numerous studies have demonstrated an early and significant activation of pancreatic NF-κB when acute experimental pancreatitis is induced in rats or mice using agents such as cerulein, taurocholate, and bile-pancreatic duct ligation [9, 11–13]. Acinar cells are considered to play a key role especially

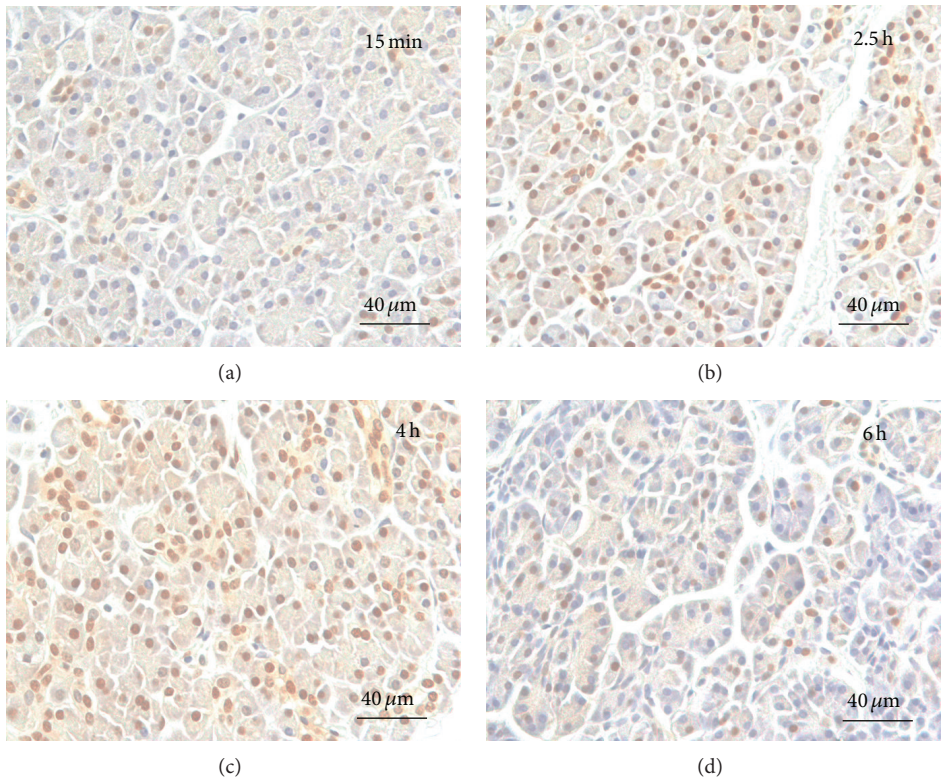

Figure 1: NF-κB activation in acinar-cell-rich pancreas. Immediately after sampling, parallel portions of each tissue specimen were immersed in saline and kept at room temperature for 15 minutes (a), 2.5 hours (b), 4 hours (c), or 6 hours (d), after which they were fixed and processed for immunohistochemical analysis. The slides were counterstained with hematoxylin. Slight staining of acinar cell nuclei can be seen at 15 minutes (a) and significant amplification is observed at 2.5 hours (b). Almost every acinar cell nucleus is stained at 4 hours (c) and activation decreases at 6 hours (d).

in early (within 30 minutes) pancreatic NF-κB activation in experimental acute pancreatitis [11]. Activation of NF-κB is followed by an increased number of proinflammatory cytokines and influx of inflammatory cells into the pancreas, leading to exacerbation of pancreatitis [9]. The importance of NF-κB in the inflammatory process is substantiated by the fact that inhibiting its activation using antioxidants (e.g., *N*-acetylcysteine) or anti-inflammatory agents (e.g., peroxisome proliferator-activated receptor γ, PPARγ) has been shown to reduce the severity of pancreatitis in animal models [9, 15, 16, 18].

MCP-1 has been associated with several inflammatory diseases, including pancreatitis. Monocytes, T-lymphocytes, acinar cells, and stellate cells have all been shown to express MCP-1, and MCP-1 has been seen to upregulate in acute and chronic pancreatitis [10]. Acini express MCP-1 as early as 60 minutes after induction of acute experimental pancreatitis [14]. The importance of MCP-1 in the pathogenesis of pancreatic inflammation was substantiated in a study by Zhao et al. [19], where pancreatic inflammation and fibrosis was significantly reduced in rats with experimental chronic pancreatitis by giving them antichemokine gene therapy. In a study by Ishibashi et al. [16] the severity of acute pancreatitis was attenuated by blocking MCP-1 activity in rat models.

Knowledge about the role of acinar cells in the pathogenesis of acute pancreatitis has progressed over recent years. It has been suggested that acinar cells can act in the same manner as inflammatory cells. The latest studies show that the acini may be promoters of the inflammatory cascade. They secrete cytokines, chemokines, and adhesion molecules, resulting in activation and recruitment of circulating leukocytes [20, 21].

The consistency of the pancreas has been shown to affect the risk of post-PD complications. A soft pancreas and a small pancreatic duct diameter are known to increase morbidity [22, 23]. Postoperative pancreatitis, or subclinical pancreatic irritation, has recently been noted as a precursor of postoperative complications such as delayed gastric emptying and postoperative pancreatic fistula [3, 6]. In animal models, any injury to pancreatic parenchyma with scalpel or sutures has been shown to initiate an inflammatory process in the parenchyma that spreads throughout the pancreas [24, 25]. In our previous study, patients with acinar-cell-rich pancreas developed massive postoperative inflammation that exposed them to clinically significant complications [6]. So as far as we know, molecular-level events related to post-PD pancreatitis in the remnant of pancreas after PD have not been studied before.

In the postoperative state the pancreatic remnant suffers from hypoxia to some extent but not from *total* ischemia as in our ex vivo study. We recognize that this study therefore does

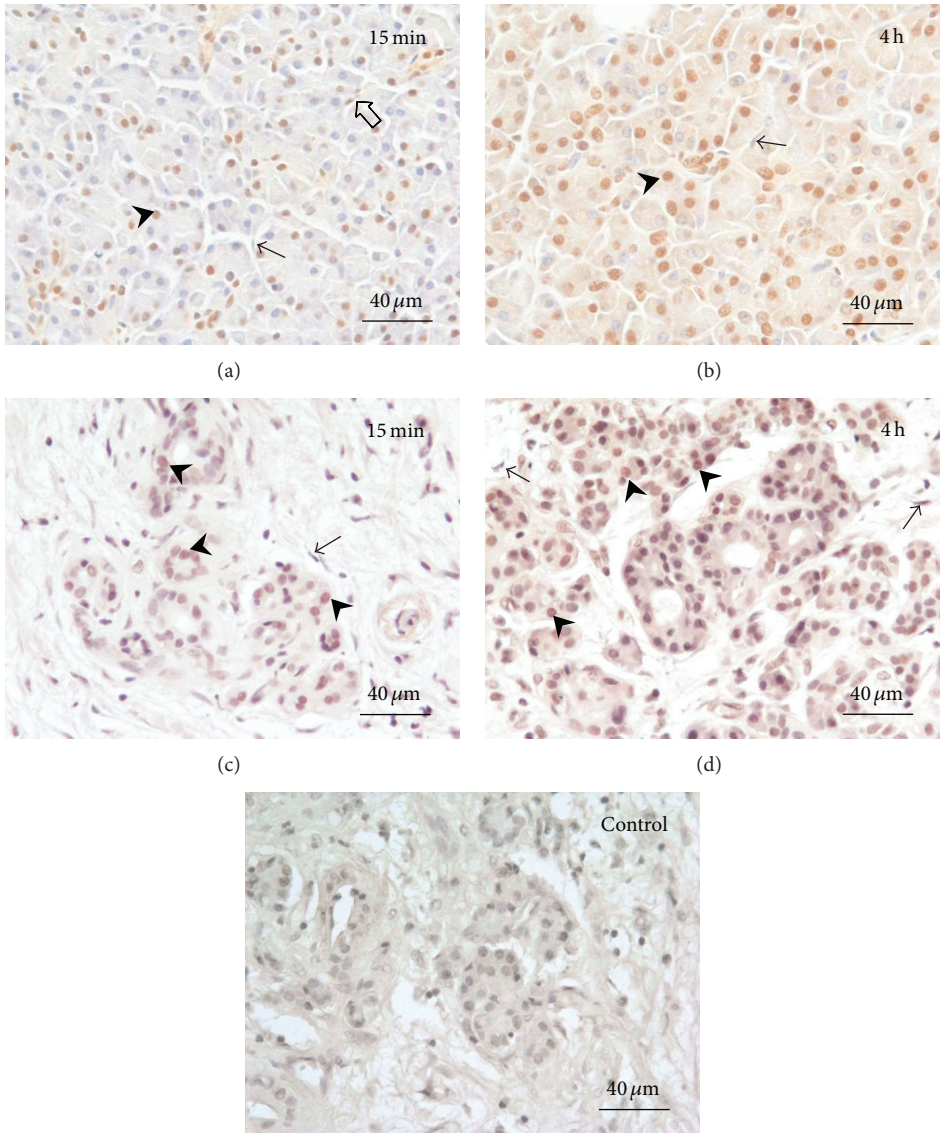

Figure 2: NF-κB expression in acinar-cell-rich ((a), (b)) and fibrotic ((c), (d)) pancreata. (a) and (c) are 15-minute sample and (b) and (d) represent 4-hour time points. Arrowheads indicate representative NF-κB-expressing nuclei in acinar cells. NF-κB-positive fibroblasts were rare (open arrow in (a)), the fibroblast nuclei being predominantly negative (arrows). The increase in NF-κB activation is more prominent in acinar-cell-rich pancreata ((a) and (b)) than in fibrotic pancreata ((c) and (d)). Control stainings were negative (e).

not perfectly mimic postoperative conditions in the patient. Hypoxia has been shown to be an independent inducer of acute pancreatitis [26] and presumably acts as an aggravating factor for surgically induced pancreatic inflammation. The intensity of acinar cell activation may therefore be magnified in this setting. Hypoxia-induced acinar cell necrosis may also explain the decreased activation of NF-κB at 6 h samples (Figure 1(d)), which is why we decided to use 4 h samples in our quantitative analyses.

In this study we found that in the acinar-cell-rich pancreata, acinar cell NF-κB and MCP-1 activation increased from mild at 15 minutes to high after the first 4 hours, and ductal MCP-1 expression was highly intense at both time points. In the fibrotic pancreata, acinar cell expression of NF-κB and MCP-1 and also ductal cell expression of MCP-1 were detected at the 6-hour monitoring, but the tissue expression of these markers remained lower. Our findings of the limiting role of fibrosis in pancreatic inflammation are also in line with a recent study of Acharya and colleagues [27], where fibrosis was seen to reduce acinar cell necrosis among patients with acute-on-chronic pancreatitis.

Whether and how fast the inflammation exacerbates into clinically relevant pancreatic inflammation or even pancreatitis are not known. However, in our previous study we showed that it is patients with acinar-cell-rich pancreas who develop clinically relevant pancreatic inflammation [6].

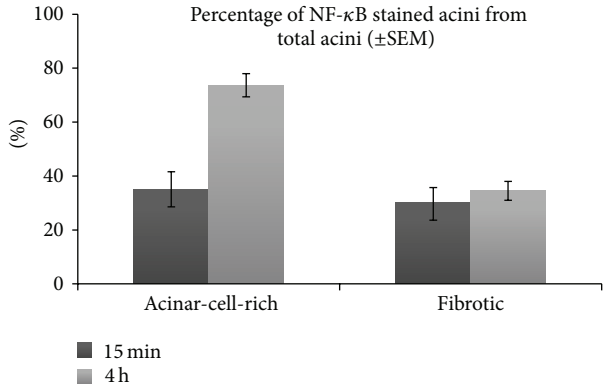

Figure 3: Comparison of NF-κB activation in acinar-cell-rich and fibrotic pancreata. The means (±SEM) of the three acinar-cell-rich and the three fibrotic samples were calculated and then compared at 15 minutes and 4 hours. In acinar-cell-rich pancreata, a significant increase in NF-κB expression occurs between 15 minutes (35%) and 4 hours (74%). In fibrotic pancreata, the change between 15 minutes (30%) and 4 hours (35%) is minor.

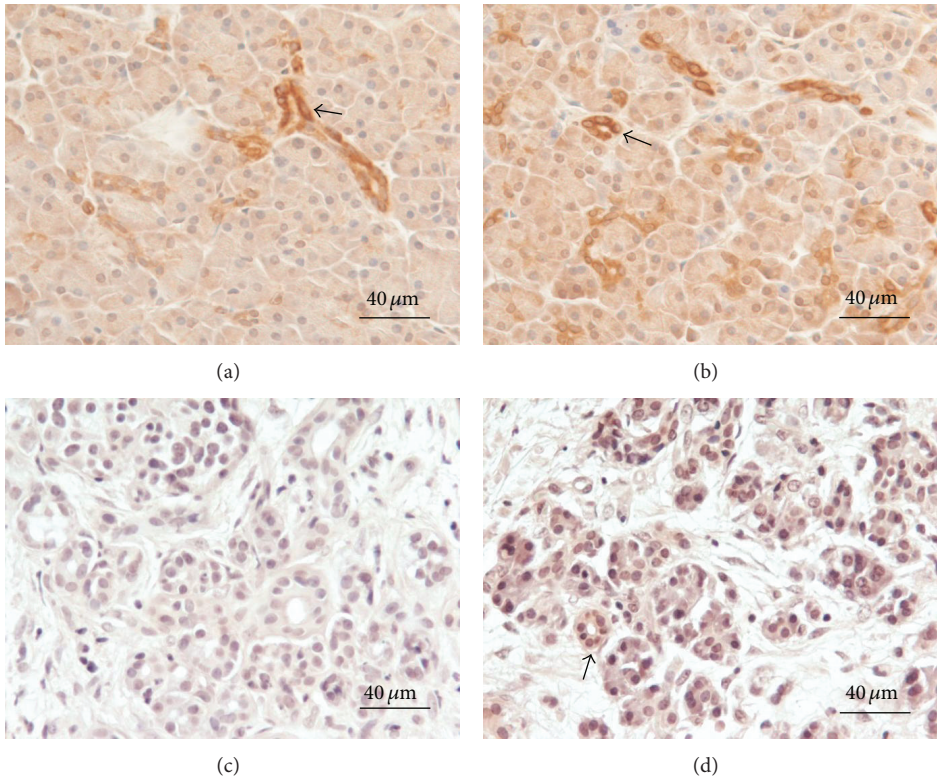

Figure 4: MCP-1 expression in acinar-cell-rich ((a), (b)) and fibrotic ((c), (d)) pancreata. Immediately after sampling, parallel portions of each tissue specimen were immersed in saline and kept at room temperature for 15 minutes ((a), (c)) or 4 hours ((b), (d)), after which they were fixed and processed for immunohistochemical analysis. MCP-1 staining was equally intense in the ductal cells of acinar-cell-rich pancreata after 15 minutes and 4 hours ((a), (b), arrows), whereas intra-acinar MCP-1 expression was observed to slightly increase with time. In fibrotic pancreata, MCP-1 in ductal and acinar cells remained undetectable at 15 minutes (c). At 4 hours, weak staining can be detected in ductal cells ((d), arrow). The slides were counterstained with hematoxylin.

5. Conclusions

We hypothesize that a patient undergoing PD who has a large amount of acinar cells in the transection line (i.e., in the pancreatic remnant) is at high risk of developing a massive postoperative inflammatory cascade in the pancreas. The first 4 hours after the induction of surgical trauma may play an important role in the patient's postoperative prognosis. As postoperative pancreatitis often precedes other complications after PD, future therapeutic strategies targeting

postoperative complications could consider anti-inflammatory treatments and could also focus them on perioperative—not just postoperative—treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Neoadjuvant Therapy in Pancreatic Cancer: An Emerging Strategy

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Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths among men and women, being responsible for 6% of all cancer-related deaths. Surgical resection offers the only chance of cure, but only 15 to 20 percent of cases are potentially resectable at presentation. In recent years, increasing evidences support the use of neoadjuvant strategies in pancreatic cancer in patients with resectable pancreatic cancer as well as in patients with borderline resectable or locally advanced PDAC in order to allow early treatment of micrometastatic disease, tumour regression, and reduced risk of peritoneal tumour implantation during surgery. Furthermore, neoadjuvant treatment allows evaluation of tumour response and increases patient's compliance. However, most evidences in this setting come from retrospective analysis or small case series and in many studies chemotherapy or chemoradiation therapies used were suboptimal. Currently, prospective randomized trials using the most active chemotherapy regimens available are trying to define the real benefit of neoadjuvant strategies compared to conventional adjuvant strategies. In this review, the authors examined available data on neoadjuvant treatment in patients with resectable pancreatic cancer as well as in patients with borderline resectable or locally advanced PDAC and the future directions in this peculiar setting.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease and still continues to have the worst prognosis of all gastrointestinal malignancies. Despite considerable advances in radiological techniques, it often presents as a locally advanced or metastatic disease in most patients and only about 10–20% of patients are considered candidate to surgery [1]. However, even in patients undergoing radical resection, the prognosis remains poor with a 5-year survival rate around 15–20% and a median overall survival (OS) in the order of 20–24 months due to the high rate of relapse [2]. In large series a 92% rate of disease relapse has been reported after PDAC resection without postoperative treatment. In particular, local recurrence occurs in about 40% of patients while distant metastases are observed as the only site of relapse in about 50% of cases, with liver as the primary site of distant relapse (36%) [3]. Margin involvement (R1) has been shown to be associated with poor prognosis in resected PDAC patients. Even in recent series a 15–35% rate of R1 resections has been reported, while macroscopically involved margins (R2) have been described in less than 1% of resections [4]. In spite of the improved outcomes observed after pancreaticoduodenectomy in the last years in high volume centers, the incidence of postoperative complications remains high (20–70%) while the mortality rate is between 1 and 4% [5].

Therefore surgery alone cannot be considered the optimal therapy for localized PDAC and complementary treatment; for example, chemotherapy and radiotherapy have been evaluated in the context of a multimodal approach.

Randomized studies have demonstrated the efficacy of adjuvant chemotherapy in patients with resected PDAC. Postoperative chemotherapy with gemcitabine provides a modest but significant survival benefit with a median OS of 23 months compared to about 20 months in patients undergoing resection alone and a 5-year survival rate of 21% versus 9% [3]. Similar results have been observed in clinical trials

with adjuvant 5-fluorouracil [6]. On the contrary, data about adjuvant chemoradiotherapy in PDAC are more controversial. A few clinical trials have evaluated chemoradiation after surgery with conflicting results ranging from a significant improvement in OS [7] to a detrimental effect [6].

Overall, these results show how postoperative therapy can provide a significant but modest benefit to PDAC patients and highlight the need for more effective multimodal treatment strategies. Although the use of preoperative treatment, such as neoadjuvant chemotherapy or chemoradiotherapy, may represent an effective strategy for localized PDAC, limited evidences are currently available on this approach.

In this review we will overview available data on neoadjuvant treatment in patients with resectable pancreatic cancer as well as in patients with borderline resectable or locally advanced PDAC.

2. Neoadjuvant Therapy in Resectable Pancreatic Cancer

Neoadjuvant treatment has a strong rationale in pancreatic cancer and presents many theoretical advantages. Indeed, preoperative chemotherapy approach allows an early treatment of micrometastatic disease, responsible for relapse after curative surgery. Furthermore, a larger proportion of patients are able to complete the treatment in the preoperative compared to adjuvant setting. In fact, it has been shown by adjuvant trials that up to 25% of patients submitted to pancreatic resection do not receive the planned treatment due to postoperative complications, deterioration of performance status, comorbidities, or early recurrence [8]. Preoperative chemotherapy may also induce tumour regression, reducing the risk of R1 resection. Other potential advantages include better patients' tolerance to chemotherapy, a reduced risk of peritoneal tumour implantation during surgery, and the chance of an *in vivo* assessment of tumour chemosensitivity. Finally neoadjuvant treatment allows a better patient selection identifying those patients presenting with rapid progressive or disseminated disease at restaging who therefore have a very poor prognosis and for whom surgery is unlikely to provide any benefit.

During the last two decades, several studies have evaluated the role of neoadjuvant chemotherapy, radiotherapy, or combination of both in resectable pancreatic cancer.

Regarding chemotherapy, a phase II randomized trial allocated 50 patients with resectable PDAC to gemcitabine alone or to gemcitabine cisplatin. A 4% response rate was observed in patients treated with combination chemotherapy while no objective response was seen in patients treated with gemcitabine alone. Resection rate was higher in patients in the combination arm (70%) compared to patients in the gemcitabine arm (38%), while there were no differences in terms of surgical complications. Even overall survival (OS) was better in combination arm (15.6 months) compared with a median OS of 9.9 months in monotherapy arm [9]. In 28 resectable PDAC patients, receiving cisplatin and gemcitabine [10], a 89% rate of resectability was observed with 71% of R0 resections, not significantly different from the rate reported in the literature with surgery alone, and a median OS of 19.1 months for patients who underwent successful resection.

More data are available regarding neoadjuvant chemoradiotherapy. Indeed, most of the trials are single phase II studies. The administration of radiation therapy before surgery has some advantages compared to the postoperative setting and in particular it allows radiation to be delivered to well oxygenated cells before surgical devascularization. In a study performed at MD Anderson, 28 patients with resectable PDAC were treated with chemotherapy with 5-FU (300 mg/m^2/day) concomitant to radiotherapy (50.4 Gy in 5.5 weeks). Gastrointestinal toxicity required hospitalization in 9 patients (32%) but no patients experienced a delay in surgery. A total of 23 patients without evidence of progressive disease underwent laparotomy and 17 patients (61%) a radical pancreatoduodenectomy. Perioperative complications occurred in three patients with one perioperative death [11]. Subsequently, the same group evaluated neoadjuvant chemoradiotherapy with gemcitabine concomitant to radiation therapy (30 Gy) on 86 patients with localized pancreatic cancer. The treatment included chemotherapy with gemcitabine (400 mg/m^2 once a week for 7 weeks) concomitant to radiotherapy (30 Gy in 10 fractions in weeks 2 and 3). Pancreaticoduodenectomy was performed in 64 patients (75%) and 57 patients (66%) had R0 resection. Median OS for the whole patients population was 22.7 months while patients who underwent surgery had a median OS of 34 months. Main grade 3-4 toxicities observed included neutropenia, fatigue, nausea, and vomiting; there was no toxic death and all the patients concluded the planned treatment [12]. A phase II trial evaluated the combination of cisplatin and gemcitabine followed by gemcitabine-based chemoradiotherapy in 90 patients with resectable PDAC. Sixty-two (78%) of 79 patients who completed chemoradiation were taken to surgery and 52 (66%) of 79 underwent PD. Interestingly, margin involvement was described only in one patient, with a R1 resection rate (4%) significantly lower compared to data reported with surgery alone in the literature. The median OS of all 90 patients from the date of diagnosis was 17.4 months (95% CI, 14.5 to 20.3 months) while patients who completed chemoradiation and underwent surgery had a median OS of 31 months [13].

Also paclitaxel in combination with radiotherapy has been tested in patients with resectable PDAC. In a study on 35 patients, paclitaxel (weekly 3 h infusion of 60 mg/m^2 for 3 consecutive weeks) concomitant to radiotherapy (30 Gy) was administered before surgery. Only 12 patients (34%) underwent R0 resection with a median OS of 19 months for the whole group and a high rate of distant failure (85%). On the whole, the results were less promising compared to what was observed with 5-FU based therapy [14]. Recently, results of a phase II trial evaluating gemcitabine and S-1 in this setting in an Asian population have been presented. The study included 36 resectable and borderline resectable PDAC patients treated with gemcitabine given at a dose of 1000 mg/m^2 on days 1 and 8 of each cycle and S-1 administered orally at a dose of 40 mg/m^2 twice daily for the first 14 consecutive days followed by a 7-day rest. The 2-year

Table 1: Comparison of definitions of borderline resectable pancreatic cancer.

	AHBPA/SSAT/SSO	MD Anderson	NCCN 2012
SMV-PV	Abutment, encasement, or occlusion	Occlusion	Abutment with impingement and narrowing
SMA	Abutment	Abutment	Abutment
CHA	Abutment or short-segment encasement	Abutment or short-segment encasement	Abutment or short-segment encasement
Celiac trunk	No abutment or encasement	Abutment	No abutment or encasement

SMV-PV: superior mesenteric vein-portal vein; SMA: superior mesenteric artery.
CHA: common hepatic artery.

survival rate, primary endpoint of the study, was 45.7%. The R0 resection rate was quite high (87%) while the perioperative morbidity was 40%, in line with data from surgical series and then with no apparent increase in complication rates compared to surgery alone [15].

Overall, these studies showed that neoadjuvant chemoradiotherapy is a feasible approach and does not increase the risk of perioperative morbidity and mortality. Furthermore, patients who completed neoadjuvant chemoradiation and did not progress at restaging had a higher chance of achieving R0 resection and a higher overall survival when compared to historical data. Nevertheless, although chemoradiation has been shown to improve local control, it may not effectively decrease distant metastasis, as shown by the high rate of distant failure in these studies.

3. Neoadjuvant Therapy in Borderline Resectable and in Locally Advanced PDAC

As previously stated, only 10–20% of PDAC patients present with primarily resectable disease while locally advanced, nonmetastatic pancreatic cancer is seen in about 30% of patients [1] and median OS in this subgroup of patients is in the order of 9–13 months. Recently this group has been further subdivided by different authors into borderline resectable (BRPC) and locally advanced nonresectable (LAPC) pancreatic cancer.

3.1. Borderline Resectable Pancreatic Cancer: Definitions. Borderline resectable cancers have been *recently* defined as cancers with limited involvement of the mesenteric vessels, such that resection is technically possible, but which carry a high risk of margin-positive resection and consequently a higher risk of recurrence [22]. Therefore these tumours are distinct from both locally advanced and unresectable tumors, which are unlikely to be resectable to negative margins despite the use of induction therapy or complex reconstructive surgical techniques that from resectable tumors that are candidates for upfront pancreaticoduodenectomy. In this scenario, preoperative treatment may be specifically beneficial in borderline resectable PDAC, improving the fraction of patients undergoing radical resection.

Several anatomic definitions of borderline resectable pancreatic cancer have been given; however, the 3 most commonly cited definitions are those proposed by the MD Anderson group and the Americas Hepatopancreatobiliary Association (AHPBA)/Society for Surgery of the Alimentary Tract (SSAT)/Society of Surgical Oncology (SSO, modified by the NCCN) (Table 1).

The definition of borderline resectable tumours according to the NCCN guidelines includes the following characteristics: (i) no distant metastases; (ii) venous involvement of the superior mesenteric vein (SMV) or portal vein demonstrating tumour abutment with impingement and narrowing of the lumen, encasement of the SMV/portal vein, but without encasement of the nearby arteries, or short-segment venous occlusion resulting from either tumour thrombus or encasement, but with suitable vessel proximal and distal to the area of vessel involvement, allowing for safe resection and reconstruction; (iii) gastroduodenal artery encasement up to the hepatic artery with either short-segment encasement or direct abutment of the hepatic artery, without extension to the celiac axis; (iv) tumour abutment of the superior mesenteric artery (SMA) not to exceed >180° of the circumference of the vessel wall. By contrast, nonmetastatic pancreatic tumours are considered nonresectable if the following characteristics are fulfilled: (i) >180° SMA or celiac artery encasement; (ii) unreconstructable SMV/portal vein occlusion; (iii) aortic invasion or encasement; (iv) metastases to lymph nodes beyond the field of resection [23]. In addition to these radiological criteria, Katz et al. [24] introduced also patient-related factors in the concept of BRPC and proposed a classification in three subgroups: group A, defined by radiological criteria; group B, including patients with findings suggestive of metastases; and group C, including patients with comorbidities and marginal performance status.

3.2. Neoadjuvant Therapy in BRPC and LAPC. The optimal neoadjuvant therapy in patients with BRPC and LAPC remains a matter of debate due to the lack of randomized studies. In both categories local tumour reduction and systemic control represent primary goals of treatment and then common strategies may be applied in both entities. Moreover, in many studies these two entities have been evaluated together.

Chemoradiotherapy is a common experimental approach therapy in BRPC. Massucco et al. [25] evaluated 28 patients with BRPC and nonresectable pancreatic cancer who received gemcitabine-based chemoradiotherapy. While only 1 out of 8 patients with initially unresectable disease underwent resection, 7 out of 18 (39%) of BRPC were resected. Chemoradiotherapy did not increase perioperative

morbidity and mortality. Median overall survival for the whole group was 15 months. In both groups, a disease-free survival beyond 24 months was observed in patients resected with negative margins. A different strategy was evaluated by Patel et al. [26] on seventeen patients with BRPC. The patients were treated with three cycles of induction chemotherapy with gemcitabine, docetaxel, and capecitabine followed by 5-FU based chemoradiotherapy with IMRT. Eleven patients (64.7%) out of 17 underwent resection and eight patients (47%) achieved an R0 resection. The median progression-free survival and OS were 10.48 months and 15.64 months, respectively. Stokes et al. [27] also prospectively examined 40 borderline resectable pancreatic cancer patients treated with combined capecitabine-based chemoradiation. Of these, 34 (85%) completed neoadjuvant treatment and were restaged. A total of 16 patients (46%) proceeded to surgery, with 88% with an R0 resection and median overall survival of 23 months. Kim et al. evaluated a chemoradiotherapy regimen including gemcitabine and oxaliplatin on 68 BRPC and LAPC patients: after the treatment, completed by 90% of patients, forty-three patients underwent resection (63%), and R0 resection was achieved in 36 of those 43 patients (84%). The median overall survival was 18.2 months for all patients and 27.1 months for those who underwent resection [28].

The benefit of neoadjuvant therapies in BRPC was retrospectively reviewed by Katz et al. [24] at MDACC. Between 1999 and 2006, 160 (7%) of 2454 pancreatic cancer patients were classified as having borderline resectable disease and were scheduled to receive 2–4 months of neoadjuvant chemotherapy followed by radiation in combination with either 5-fluorouracil (5-FU), gemcitabine, capecitabine, or paclitaxel. Restaging CT scan was repeated every 2 months during the treatment and 4 to 6 weeks after completion to determine resectability. Patients who experienced disease progression or had deterioration of performance status during this period of treatment were excluded from surgery. One hundred twenty-five (78%) patients completed the restaging, 79 (63% of 125) patients proceeded to surgery, and 66 (53% of 125) patients received pancreaticoduodenectomy. For the whole patients population (160 patients) with borderline resectable disease, the median OS was 18 months and the 5-year survival was 18%. Importantly, the 66 patients who completed the whole therapy including surgery had a significantly better clinical outcome (median OS of 40 months and a 5-year survival rate of 36%) compared to a median survival of 13 months in the remaining 94 unresected patients. Patients with greater pathologic response or drop in serum CA19-9 levels during neoadjuvant therapy had better OS. However, 59% of the resected patients had a recurrence, mainly occurring in distant organs such as lung, liver, or bone (45%); 9% had recurrence in the pancreatic bed; and 11% had recurrence in the peritoneum or regional lymph nodes. These results confirm a positive effect of neoadjuvant treatment in terms of resection rates and long-term survival in patients with BRPC. However, the high rates of disease relapse claim for more effective treatments.

Data about the efficacy of chemotherapy in LAPC mainly come from subgroup analysis of studies in advanced pancreatic cancer. Most of the studies investigating the efficacy of gemcitabine-based chemotherapy in advanced pancreatic cancer included a percentage of LAPC patients. Gemcitabine-based combinations have proved to induce higher response rate (about 26%) compared to single agent gemcitabine (4–15%) and response rates were similar to those observed in metastatic disease [29, 30]. A phase II trial, the NeoGemOx trial, evaluated gemcitabine and oxaliplatin combination in 33 LAPC patients. After treatment, 39% of patients underwent curative resection, with a 69% of R0 resections. Median OS of patients who underwent tumor resection was 22 months compared with 12 months for those without resection ($P = 0.046$). The study confirmed that the combination of gemcitabine and oxaliplatin is active in LAPC patients and induces tumour regression in a significant proportion of patients [31]. Also the combination of gemcitabine and capecitabine has been assessed in this subset of patients. In a study by Lee et al., forty-three patients (18 with BRPC and 25 with unresectable disease) were treated with fixed-dose rate gemcitabine and capecitabine. Surgery was performed in 17 patients (39.5%); pathologic radical resection (R0) was achieved in 82.3% among the 17 resected patients. Median OS was 23.1 months in patients undergoing surgery [32]. An Italian study evaluated an upfront intensive chemotherapy combination followed by chemoradiotherapy in the treatment of LAPC. In particular, patients received PEFG/PEXG (cisplatin, epirubicin, 5-fluorouracil/capecitabine, and gemcitabine) or PDXG (docetaxel substituting epirubicin) regimen for 6 months followed by radiotherapy (50–60 Gy) with concurrent gemcitabine or fluoropyrimidines. A high response rate was observed (47%) while stable disease was reported in 42% of patients. Thirteen patients of ninety-one included in the analysis (14%) were radically resected yielding one pathologic complete remission [33]. The use of CRT alone in LAPC has been evaluated in different studies. One randomized study demonstrated the superiority of 5-fluorouracil (5-FU) based CRT compared with best supportive care. Most studies used 5-FU or gemcitabine as reference chemotherapy in combination with radiation doses of 50–60 Gy. A recent meta-analysis suggested that the combination of radiation with gemcitabine might be more effective than the combination with 5-FU [34].

A recent systematic review has evaluated 111 trials including 4394 pancreatic cancer patients [35]. Neoadjuvant therapy included chemotherapy in 96% and radiation therapy in 94% of studies. For nonresectable patients, the estimated overall response rate was 35%. Among patients with initially nonresectable tumours, surgical exploration was performed in 47% of cases. The overall resection rate after neoadjuvant therapy was 33%, and 79% of resections were R0 resections. Median OS of the 33% resected patients was 20.5 months while unresectable patients, who were not resected, had a median OS of 10.2 months. This analysis suggests that neoadjuvant treatment may be able to induce conversion to resectability in about one-third of LAPC patients and, importantly, tumour resection is associated with significantly prolonged overall survival, comparable to what was observed in primarily resectable pancreatic cancer patients.

Table 2: FOLFIRINOX regimen in patients with borderline resectable or locally advanced unresectable pancreatic cancer.

Author	Study design	*n* pts	ORR	Resection rate	R0 resections	1-year PFS
Hosein et al. [16]	Retrospective	18	—	39%	28%	83%
Gunturu et al. [17]	Retrospective	16	50%	—	—	—
Peddi et al. [18]	Registry study	23	34%	—	—	75%
Blazer et al. [19]	Retrospective	43	—	54%	42%	—
Vasile et al. [20]	Phase II	32	37%	41%	—	—
Kunzmann et al. [21]	Phase II*	8	63%	37%	—	—

* Sequential regimen including FOLFIRINOX and nab-paclitaxel plus gemcitabine.

3.3. New Treatment Strategies. Recently FOLFIRINOX, a three-drug combination regimen including oxaliplatin, irinotecan, leucovorin, and 5-FU, was shown to be superior to gemcitabine in patients with metastatic pancreatic cancer, with a median OS of 11.1 months in the FOLFIRINOX group versus 6.8 months in the gemcitabine group ($P < 0.001$) and a median progression-free survival of 6.4 months versus 3.3 months ($P < 0.001$), although FOLFIRINOX toxicity was higher [36]. Interestingly, FOLFIRINOX demonstrated also a higher response rate compared to gemcitabine (32% versus 9%, $P < 0.001$). Notably, the phase III study by Conroy et al. included only patients with metastatic disease and then results cannot be translated to LAPC patients. However, preliminary data on the efficacy of FOLFIRINOX regimen in LAPC patients are available from some retrospective analysis or small case series (Table 2).

A retrospective analysis conducted by Hosein et al. [16] on 18 patients with borderline resectable and unresectable pancreatic cancer showed a 28% rate of R0 resection after chemotherapy. Among the 11 patients who remained unresectable after FOLFIRINOX, 3 went on to have R0 resections after combined chemoradiotherapy, for an overall R0 resection rate of 44%. An analysis reported by Gunturu et al. [17] included 16 patients with LAPC treated with FOLFIRINOX. A response rate of 50% was shown in LAPC patients; interestingly, the authors modified the FOLFIRINOX regimen with dose reductions in 29 out of 35 patients starting from the first cycle. This dose attenuation of irinotecan and bolus fluorouracil was shown to improve tolerability without compromising efficacy. Similar results were reported by Peddi et al. [18] in a multi-institutional registry study. Among 23 patients with BRPC and LAPC a 34% response rate was observed, with an 84% disease control rate, in spite of dose reductions. In particular deletion of 5-FU and dose reduction of irinotecan were the most common modifications applied.

More recently, results of a retrospective analysis conducted on 43 patients with BRPC and LAPC treated with a modified FOLFIRINOX regime have been presented [19]. The regimen used in the study had no bolus 5-FU and a lower dose of irinotecan compared to the regimen evaluated by Conroy. Overall resection rate was 53.8% including 45% of patients with initially unresectable disease and R0 resection was achieved in 85.7% of the resected patients. The median PFS of resected patients in this analysis reached 18.4 months. All patients received prophylactic pegfilgrastim and rate of G3/4 hematological toxicities was remarkably low with no episode of febrile neutropenia or G3/4 thrombocytopenia. A similar chemotherapy combination, the FOLFOXIRI regimen, has been evaluated in an Italian phase II study in 32 patients with unresectable or borderline resectable PDAC patients. FOLFOXIRI consisted of a lower dose of irinotecan (150 mg/m^2) and of infusional 5-fluorouracil (2800 mg/m^2 as a 48-hour continuous infusion on days 1 to 3) compared to FOLFIRINOX with no bolus 5-fluorouracil, while folinic acid and oxaliplatin (85 mg/m^2) remained unchanged. The FOLFOXIRI regimen was active, with a 37% objective response rate, and allowed radical resection in 41% of patients with a median OS for the patients enrolled of 24.2 months [20].

Nab-paclitaxel, an albumin bound formulation of paclitaxel particles, has recently been shown to be effective in the treatment of advanced pancreatic cancer. The MPACT trial, comparing nab-paclitaxel and gemcitabine versus gemcitabine alone, found a significant increase in median OS (8.5 months versus 6.7, $P < 0.001$) in PFS (median PFS 5.5 months versus 3.7 months, $P < 0.001$) and in overall response rate (23% versus 7%, $P < 0.001$) for the combination [37]. This trial included only patients with metastatic disease; therefore data about the efficacy of nab-paclitaxel in patients with locally advanced disease are lacking. However, considering the hypothesis that the antitumour effect of nab-paclitaxel is mediated by depletion of peritumoural stroma and improved transport of chemotherapeutic agents to the tumour, the use of this drug in localized disease in the neoadjuvant setting appears to be promising. In 2013, preliminary results of a pilot study of sequential neoadjuvant chemotherapy with nab-paclitaxel plus gemcitabine followed by FOLFIRINOX in locally advanced pancreatic cancer were presented [21]. Eight LAPC patients received 2 cycles of nab-paclitaxel and gemcitabine followed by 2 cycles of FOLFIRINOX. All patients received the planned 4 cycles of neoadjuvant chemotherapy without dose reductions and there were no treatment-related deaths and none of the patients stopped treatment due to toxicity. Among the 8 evaluable patients, 5 partial responses (63%) and 3 stable disease (37%) were observed, resulting in a disease control rate of 100%. After sequential chemotherapy 3 patients (37%) underwent radical surgical resection. Notably, all resected tumours showed signs of tumour regression with one patient showing a complete pathological response.

Although data about the efficacy of new treatment regimens in neoadjuvant setting are promising, prospective studies are required to confirm the efficacy and the tolerability of FOLFIRINOX and nab-paclitaxel in BRPC and LAPC.

4. Ongoing Trial in Neoadjuvant Setting for PDAC

Among the 1607 studies running in patients with pancreatic cancer, 91 studies are focused on neoadjuvant chemotherapy. A selection of these trials is summarized in Table 3. Several studies are assessing the efficacy of gemcitabine alone or in combination with other drugs in this setting. Among them, a multicenter prospective randomized phase II/III study of neoadjuvant chemoradiation with gemcitabine is ongoing in patients with borderline resectable pancreatic cancer. This study is designed in 2 arms, one with upfront surgery and the other with neoadjuvant chemoradiation therapy (NCT01458717).

Another randomized phase III study (NEOPAC study, NCT01314027) is comparing adjuvant gemcitabine and neoadjuvant gemcitabine/oxaliplatin plus adjuvant gemcitabine in resectable pancreatic cancer. On the other hand, the NEOPA study is a phase III trial of chemoradiation with weekly gemcitabine 300 mg/m^2 for 6 weeks combined with external beam radiotherapy (EBRT) followed by surgery and adjuvant gemcitabine (1000 mg/m^2 6 cycles at days 1, 8, and 15 of each 28-day cycle) versus upfront surgery followed by adjuvant gemcitabine (NCT01900327).

Moreover, a phase I study is evaluating the association of neoadjuvant hypofractionated chemoradiation with gemcitabine plus radiosurgical boost for patients with borderline resectable and locally advanced unresectable pancreatic cancer (RT-054, NCT01739439).

Furthermore, a phase I study of preoperative gemcitabine plus CD40 agonist antibody CP-870,893 followed by addition of CP-870,893 to standard of care adjuvant chemoradiation is recruiting patients with newly diagnosed resectable PDAC (NCT01456585).

Gemcitabine is also under evaluation in a phase II study in combination with capecitabine followed by SBRT for potentially resectable, locally advanced PDAC (NCT01360593) and in a phase II study in combination with erlotinib before and after pancreatectomy for patients with operable PDAC (NCT00733746).

Abraxane represents one of the most promising agents for patients with pancreatic cancer. With regard to its use in the neoadjuvant setting, the NEONAX randomized phase II trial is in course to compare neoadjuvant plus adjuvant or only adjuvant nab-paclitaxel plus gemcitabine for resectable pancreatic cancer (NCT02047513). This combination is under evaluation also in an open-label phase 1/2 study that will combine gemcitabine and nab-paclitaxel with an oral hedgehog inhibitor LDE225 in patients with borderline resectable PDAC (NCT01431794).

Presently, six studies are assessing the potential of FOLFIRINOX as neoadjuvant therapy for pancreatic cancer. Among them, a phase II trial has been designed with FOLFIRINOX (5-fluorouracil, irinotecan, oxaliplatin, and gemcitabine) for six cycles prior to combined modality treatment with gemcitabine during and following IMRT (NCT01661088).

In addition, two phase II trials are ongoing with FOLFIRINOX and chemoradiation followed by definitive surgery (NCT01677988) or definitive surgery and postoperative gemcitabine for patients with borderline resectable pancreatic adenocarcinoma (NCT01821612).

Moreover, two phase II studies are assessing the efficacy and safety of pre- and postsurgery FOLFIRINOX in patients with localized pancreatic cancer (NCT01660711, NCT02047474).

Finally, a phase I study of stereotactic body radiation therapy (SBRT) and neoadjuvant FOLFIRINOX is ongoing in resectable pancreatic cancer (NCT01446458).

Several trials are in course to evaluate the use of capecitabine as neoadjuvant therapy for pancreatic cancer patients. Among them, a phase II trial has been opened to evaluate the efficacy and safety of the combination of capecitabine, oxaliplatin, and irinotecan (CAPOXIRI) in patients with resectable, borderline resectable, and locally advanced PDAC (NCT01760252). In addition, a phase II study is investigating the role of neoadjuvant proton beam radiation therapy and concomitant capecitabine in marginally resectable carcinoma of the pancreas (NCT00763516). Another phase II study of neoadjuvant accelerated short course radiation therapy with proton beam capecitabine and hydroxychloroquine (NCT01494155) and a randomized phase II/III trial, testing the efficacy and safety of peri- or postoperative chemotherapy with capecitabine, cisplatin, epirubicin, and gemcitabine in resectable PDAC (NCT01150630), are enrolling patients. Finally, a phase II/III nonrandomized study has been designed to assess the safety and benefit of 6 cycles of chemotherapy treatment consisting of gemcitabine, capecitabine, and docetaxel (also called "GTX"). In group I, patients with only venous involvement receive 6 cycles of gemcitabine, capecitabine, and docetaxel (GTX) and then surgery. In group II, patients with arterial involvement and/or venous involvement receive 6 cycles of GTX, then GX/RT and then surgery (NCT01065870). Another phase II trial is ongoing to evaluate the efficacy and safety of SBRT in combination with GTX in patients with borderline resectable PDAC (NCT01754623).

Even immunotherapeutic approaches are considered as neoadjuvant therapies. A phase II study of neoadjuvant chemotherapy (gemcitabine and fluorouracil) with and without immunotherapy to CA125 (Oregovomab) followed by hypofractionated stereotactic radiotherapy and concurrent HIV protease inhibitor Nelfinavir is in course in patients with locally advanced pancreatic cancer (NCT01959672).

Finally, a phase II study is evaluating the combination of fluorouracil prodrug Tegafur, leucovorin, and concomitant RT with or without cetuximab in patients with locally advanced pancreatic cancer (PERU, NCT01050426). In addition, the NEOPANC trial has been designed as a prospective, one armed single center study to investigate neoadjuvant short course intensity-modulated radiation therapy (IMRT) in combination with surgery and intraoperative radiation therapy (IORT) followed by adjuvant chemotherapy in patients with primarily resectable pancreatic cancer (NCT01372735).

Table 3: Ongoing clinical trials in neoadjuvant setting in PDAC.

Treatment	Setting	Trial identification number	Phase	Design
Gemcitabine	BRPC	NCT01458717	II/III	Upfront surgery versus neoadjuvant gemcitabine-based chemoradiation therapy
Gemcitabine/Oxaliplatin	Resectable PC	NCT01314027	III	Adjuvant gemcitabine versus neoadjuvant gemcitabine/oxaliplatin plus adjuvant gemcitabine
Gemcitabine + RT	Resectable PC	NCT01900327	II	Chemoradiation with gemcitabine + RT followed by surgery and adjuvant gemcitabine versus upfront surgery plus adjuvant gemcitabine
Gemcitabine + RT	BRPC and LAPC	NCT01739439	I	Hypofractionated chemoradiation with gemcitabine plus radiosurgical boost
Gemcitabine + CP-870,893 + RT	Resectable PC	NCT01456585	I	Gemcitabine plus CD40 agonist antibody CP-870,893 followed by addition of CP-870,893 versus adjuvant chemoradiation
Gemcitabine + capecitabine	Resectable PC	NCT01360593	II	Gemcitabine + capecitabine + RT
Gemcitabine + erlotinib	Resectable PC	NCT00733746	II	Gemcitabine + erlotinib before and after surgery
Nab-paclitaxel + gemcitabine	Resectable PC	NCT02047513	III	Neoadjuvant plus adjuvant or only adjuvant nab-paclitaxel plus gemcitabine for resectable pancreatic cancer
Nab-paclitaxel + gemcitabine + LDE225	BRPC	NCT01431794	II	Gemcitabine and nab-paclitaxel with LDE225 (oral hedgehog inhibitor)
FOLFIRINOX	Resectable PC	NCT01661088	II	FOLFIRINOX followed by combined modality treatment with gemcitabine during and following RT
FOLFIRINOX	Resectable PC	NCT01677988	II	FOLFIRINOX and chemoradiation followed by surgery
FOLFIRINOX	BRPC	NCT01821612	II	FOLFIRINOX and chemoradiation followed by surgery and adjuvant gemcitabine
FOLFIRINOX	Resectable PC	NCT01446458	I	RT and neoadjuvant FOLFIRINOX
CAPOXIRI	Resectable PC, BRPC, and LAPC	NCT00087022	II	Neoadjuvant capecitabine, oxaliplatin, and irinotecan (CAPOXIRI)
Capecitabine + RT	BRPC	NCT00763516	II	Neoadjuvant proton beam radiation therapy and concomitant capecitabine
capecitabine and hydroxychloroquine + RT	Resectable PC	NCT01494155	II	Neoadjuvant accelerated short course RT with proton beam capecitabine and hydroxychloroquine
capecitabine, cisplatin, epirubicin, and gemcitabine	Resectable PC	NCT01150630	II/III	Peri- or postoperative chemotherapy with capecitabine, cisplatin, epirubicin, and gemcitabine
GTX + RT	BRPC	NCT01065870	II/III	Gemcitabine, capecitabine, and docetaxel (GTX) versus GTX + RT
GTX + RT	BRPC	NCT01754623	II	RT in combination with GTX
Gem, 5-FU Oregovomab, Nelfinavir + RT	LAPC	NCT01959672	II	Gemcitabine and 5-FU with and without immunotherapy (Oregovomab) followed by RT and Nelfinavir
Tegafur, cetuximab + RT	LAPC	NCT01050426	II	Tegafur, leucovorin, and concomitant RT with or without cetuximab
IMRT + IORT	Resectable PC	NCT01372735	II	IMRT in combination with surgery and intraoperative radiation therapy (IORT) followed by adjuvant chemotherapy

5. Conclusions

Increasing evidences support the use of neoadjuvant strategies in pancreatic cancer. Nevertheless, the role of neoadjuvant therapy in patients with resectable pancreatic cancer has not yet been defined. Most of the available evidences derive from retrospective analysis or small case series, and in many studies chemotherapy or chemoradiation therapies used were suboptimal. Prospective and controlled randomized trials using the most active chemotherapy regimens currently available are warranted to assess the benefit of neoadjuvant strategies compared to conventional adjuvant strategies in this setting. Presently, the use of neoadjuvant therapies in patients with resectable pancreatic cancer could be considered in the context of a multidisciplinary approach, within clinical trials or for patients with high risk of early relapse. In particular, it has been demonstrated that high CA 19.9 serum levels (CA 19.9 > 200 U/mL), long duration of preoperative symptoms (>40 days), and pathological grading (G3-G4) are associated with high risk of early relapse and then may be used to identify patients who may benefit from preoperative chemotherapy [38].

In patients with borderline resectable or nonresectable pancreatic cancer, neoadjuvant therapy may achieve downsizing of the tumour, increasing the probability of R0 resections, or may convert the tumour to become resectable. Moreover, in patients with BRPC neoadjuvant chemotherapy may also be able to identify a subgroup of patients with early progression and so being unlikely to benefit from surgery. Currently available data do not allow defining an optimal regimen in this setting. Combination chemotherapy appears to achieve higher response rates than single-agent chemotherapy, while there are no sufficient evidences to show that chemoradiotherapy is superior to chemotherapy alone. Further options are arising, with the development of new and more effective chemotherapeutic regimens, namely, FOLFIRINOX and nab-paclitaxel. However, the efficacy of these treatments in neoadjuvant setting needs to be verified in prospective clinical trials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

High-Intensity Focused Ultrasound Treatment for Advanced Pancreatic Cancer

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Pancreatic cancer is under high mortality but has few effective treatment modalities. High-intensity focused ultrasound (HIFU) is becoming an emerging approach of noninvasively ablating solid tumor in clinics. A variety of solid tumors have been tried on thousands of patients in the last fifteen years with great success. The principle, mechanism, and clinical outcome of HIFU were introduced first. All 3022 clinical cases of HIFU treatment for the advanced pancreatic cancer alone or in combination with chemotherapy or radiotherapy in 241 published papers were reviewed and summarized for its efficacy, pain relief, clinical benefit rate, survival, Karnofsky performance scale (KPS) score, changes in tumor size, occurrence of echogenicity, serum level, diagnostic assessment of outcome, and associated complications. Immune response induced by HIFU ablation may become an effective way of cancer treatment. Comments for a better outcome and current challenges of HIFU technology are also covered.

1. Pancreatic Cancer

Pancreas is an essential gland organ in the digestive and endocrine system, producing hormones (i.e., insulin, glucagon, and somatostatin) into the bloodstream and secreting pancreatic juice to the small intestine or gut. Although pancreatic cancer is the twelfth most common cancer for humans, its mortality ratio is as large as 98% [1] and is the fourth leading cause of cancer death. 338,000 new cases were diagnosed in 2012, and the estimated 5-year prevalence of pancreatic cancer is 4.1 per 100,000 in the world. About 55% of pancreatic cancer cases occurred in more developed countries, such as Northern America and Europe, while Africa and Asia have the lowest incidence. The American Cancer Society estimates that about 46,420 people (23,530 men and 22,890 women) will be diagnosed with pancreatic cancer and among them 39,590 people (20,170 men and 19,420 women) will die in the United States in 2014. In Europe, the corresponding death number is estimated to be 80,266 people (40,069 men and 40,197 women) [2]. A comprehensive genetic analysis of 24 pancreatic cancers showed an average of 63 genetic alterations. These alterations defined 12 core cellular signaling pathways and processes in 67 to 100% of the tumors, which suggests the complexity of pancreatic tumor's genetics [3].

Owing to the absence of specific symptoms and effective screening, most of pancreatic cancers are diagnosed at the late stage (TNM III or IV) with locally advanced (60%) and metastatic disease (20%). Only about 15 to 20% of patients can undergo curative surgical resection, and the 5-year survival is just 30%. Surgery also associates a considerable risk of morbidity and mortality. If the tumors are involved with superior mesenteric artery and/or celiac axis even in the early stages, they are also generally considered unresectable. Liver, peritoneum lungs, bones, and brain are the popular sites of metastases in pancreatic cancer sorted by their feasibilities. Metastases to muscle, skin, heart, pleura, stomach, umbilicus, kidney, appendix, spermatic cord, and prostate are occasionally observed. Gemcitabine is the gold standard drug for advanced pancreatic cancer; however, its clinical benefit response (CBR) is 12 to 23.8%, and the median survival is only prolonged by a further 10 days. Erlotinib is the only targeted drug approved by the Food and Drug Administration (FDA). Chemotherapy, radiotherapy, and targeted drug are rather

ineffective for this malignancy [4]. The median survival of pancreatic cancer patients is less than 3 months without therapy and less than 6 to 12 months with therapy. Overall 1-, 3-, and 5-year survival of pancreatic cancer patients are 16%, 5%, and 4%, respectively [5]. Therefore, alternative solution for inoperable cases is strongly desired.

Most pancreatic cancer patients have severe abdominal pain and significantly decreased quality of life, which is mainly owing to the proximity of the pancreas to the duodenum, liver, stomach, jejunum, and transverse colon. The pain is usually dull and radiates to the waist, sometimes sharp and severe and could be both neuropathic and inflammatory because of both tumor expansion and invasion of the celiac and mesenteric plexus. Sleep and appetite will be affected when an advanced tumor invades the solar plexus. Pain relief for advanced pancreatic cancer patients to enhance their quality of life is an ongoing challenge. Although an increasing number of effective opioids are available for the pain mitigation, these analgesics have obvious adverse effects, such as vomiting, constipation, and dysphoria to respiratory depression. Chemotherapy and radiotherapy are not very effective in pain relief, and the associated side effects are very serious.

Although radiofrequency ablation (RFA), percutaneous ethanol injection, cryoablation, microwave ablation, and laser-induced interstitial thermotherapy have been used widely to induce coagulative necrosis for various solid tumors, it remains difficult to use them to manage those in difficult locations, such as pancreatic malignancies. Precise ablation in advanced pancreatic cancer is necessary owing to the high propensity of complications in the surrounding pancreatic parenchyma; otherwise, a pancreaticocutaneous fistula and severe pancreatitis will be produced. So, none of them is standardized for pancreatic malignancies. RFA was used for coagulation of unresectable pancreatic cancer, but two patients died from severe complications in 20 treated cases [6].

2. HIFU Technology and Mechanism

Interaction of ultrasound at great intensity with tissue and, subsequently, physical and biological changes has been investigated for decades [8]. Therapeutic ultrasound can be classified based on the intensity; the low intensity (0.125–3 W/cm^2) is to stimulate physiological responses or to accelerate the transport of drugs across the skin while the high intensities (>5 W/cm^2) intend to selectively destroy tissue in a controlled fashion. By focusing high power ultrasound beams inside the human body away from the source, almost complete necrosis of tumor lying within the focal region, especially those in difficult locations, could be achieved successfully without damage to the intervening tissue. Ultrasound surgery was first proposed as a destroying tool for neurosurgical research [9]. In the 1950s, Fry brothers applied high-intensity focused ultrasound (HIFU) to treat 50 Parkinson's disease patients [10], and the first case of breast cancer by HIFU was reported in 1961 [11]. Ultrasound hyperthermia was utilized for the treatment of glaucoma in the 1980s [12]. The advent of clinical imaging and computer control techniques in the early 1990s made practical implementation of HIFU feasible and acceptable. In 1996, 20 cases of ablation of superficial bladder cancer using HIFU were reported [13]. Wide application of HIFU in clinics began from successful treatment on a patient with osteosarcoma in Chongqing, China, in 1997. Over the past 15 years, more than 30,000 cases of uterine fibroids and cancers in the liver, breast, pancreas, bone, and kidney have been performed using HIFU with promising results [14].

The major advantages of HIFU technology are summarized as follows, but not limited [15, 16]. HIFU is a completely noninvasive procedure. There is no requirement of incisions or transfusions in the tumor ablation; thus the risks and complications associated with invasive procedures could be minimized. Acoustic intensity is only at a high level in the focal region, but not in the intervening tissue, significantly reducing the side effects, such as skin burns, discomfort, and collateral damage (i.e., hemorrhage). A broad range of tumors could be treated if the acoustic transmission window is available. Because of no ionizing radiation involved with HIFU, theoretically, there is no limitation on the number of sessions. HIFU treatment can be performed with the patient either fully conscious, lightly sedated, or under light general anesthesia. Most importantly, HIFU offers an alternative for patients who do not have any other option available.

HIFU ablation is performed under the guidance of either magnetic resonance (MR) or ultrasound (US) imaging. Magnetically compatible HIFU transducers have been developed, and MR guidance (MRgHIFU or MRgFUS) allows cancer targeting, assessment of tissue damage, and treatment monitoring by thermometry. MRI is sometimes superior in obese patients (limited to <113 kg for the gantry), but more expensive and labor-intensive. The MR thermometry has the typical temporal frame rate of 1 to 4 seconds and spatial resolution of 2 mm × 2 mm × 6 mm. Therefore, it may be more suitable for slow heating. MRgHIFU has already been approved by the FDA for clinical therapy of uterine fibroids and breast cancer. In comparison, ultrasound-guided treatments (USgHIFU) can check the acoustic conditions in the HIFU propagation path using the same energy modality and examine the changes of echogenicity in the B-mode image in real time but cannot display the temperature maps. Elastography in sonography and MRI can measure the tissue stiffness and have the potential of assessing the lesion formation.

Pathological examination illustrates clear evidence of homogeneous coagulative necrosis, cellular destruction, pyknotic nuclei or nuclei shrink, and cell debris in the target region. The boundary between the lesion and surrounding tissue is extremely sharp, comprising only a few cell layers (~50 μm). Granulation tissue, immature fibroblasts, inflammatory cells, and new capillaries are found in the margin. Small vessels (<2 mm in diameter) in the tumor, including branches of arteries and veins, are heavily destructed, which is conformed by the disappearance of endothelial cell nuclei, indistinction of cellular margins, and disruption of junctions between individual cells. Scattered intravascular thrombi are often found in the destructed vessels. As a result, there is reduced or no blood circulation in the HIFU ablated tumor

and a thin peripheral rim of contrast enhancement around the coagulative necrosis. Thermolysis in the capillary is not as effective as in larger vessels [17].

Mechanisms of HIFU are a synergy of thermal effects, mechanical effects, and biological effects [14]. The quick temperature rise over 70°C within seconds in the focal region causes necrosis, liquefaction, and fibrosis of tissues. Although the majority of the initial cell death is due to necrosis from thermal injury, HIFU can also induce apoptosis, which is the major mechanism of cell death in hyperthermia and occurs at lower thermal dose than thermal necrosis. In apoptotic cells, the cell nucleus destructs with rapid DNA degradation by endonucleases by itself [18]. Tissue can be regarded as viscous fluid contained by membranes. When an acoustic wave propagates through it, relative displacement of tissue layers and directional motion or microstreaming of the fluid will occur. High shear forces produced by the microstreaming can cause transient damage to cell membranes. Viscous friction of different layers of fluid then results in the temperature elevation. bubble cavitation, the dynamics of a gas cavity with response to the alternating compressive and rarefractional acoustic pressure, is a common phenomenon in the ultrasound therapy. Gas cavity works as an effective enhancer for heat deposition, but higher concentration will lead to the change of lesion from a cigar shape to a tadpole with the head moving towards the source, which makes the control of ablation difficult and unpredictable despite enhanced therapy efficiency. If the temperature is close to 100°C, boiling in tissue may occur. A vaporized cavity with complete tissue lysis may be produced by the motion of the large boiling bubble but without protein denaturation [23].

HIFU ablation for patients with early stage cancer is curative, and a normal tissue margin is set to be about 1.5 to 2.0 cm. In contrast, it is palliative for those with advanced cancer, impeding tumor growth and improving the quality of life. After HIFU ablation, 5 to 10% of patients are under a mild fever (~38.5°C) for about a week. Several patients with huge hepatocellular carcinoma had severe fever as high as 39.5°C for 2 to 3 weeks. The severity and duration of fever seem to be directly related to the ablated tissue volume. 20 to 30% of patients experience slight and mild local pain within 1 week after HIFU ablation, but only 5 to 10% of them are given oral analgesics for 3 to 5 days. Nerve fiber was also damaged by HIFU on 4 cases of malignant bone tumor. However, nerve functions (e.g., sensation and motion) recovered completely in two patients and partially recovered within 1 year in the others. HIFU can relieve the tumor-origin pain, which is not well controlled by antineoplasty and pharmacology successfully in patients [24]. Altogether, HIFU can destroy tissue, kill tumor cells, restrain the malignant proliferation, reduce pain, and prevent metastasis.

3. HIFU Application on Pancreatic Cancer

HIFU has been used as a palliative approach for advanced pancreatic cancer (TNM stages II–IV) mostly in China since the late 1990s; Korea and Japan also adopted this modality; a few cases in Europe were reported but none in the USA because of no approval by the FDA now. Clinical cases were reported since 2001. There are in total 241 papers on HIFU application of advanced pancreatic cancer in clinics and review (mostly in Chinese) till 2013 as shown in Figure 1. The total number of patients treated by HIFU alone, HIFU with chemotherapy, and HIFU with radiotherapy is 3022 (77.74%), 668 (17.19%), and 197 (5.07%), respectively. Inclusion criteria usually are evidence of pancreatic cancer confirmed pathologically with either biopsy in initial laparotomy or sonography guided fine-needle biopsy or diagnosed by computed tomography (CT) or positron emission tomography (PET)/CT and serum analysis; presence of inoperable pancreatic cancer on the basis of surgical consultation or refusal to undergo pancreaticoduodenectomy or other treatments; minimum diameter of a solid tumor (≥1.0 cm); Karnofsky performance scale (KPS) score of at least 70%; adequate bone marrow (white blood cell count 42500/mL, platelet count 480,000/mL, and haemoglobin 48 g/mL), renal (serum creatinine concentration <1.5 mg/dL, blood urea nitrogen <20 mg%), and hepatic functions (serum transaminase level <2× the upper normal range) except hyperbilirubinemia due to obstructive jaundice; no palliative antitumor treatments have been performed in the previous 3 months. Exclusion criteria are the intolerance to HIFU treatment; radiotherapy or chemotherapy administered in the last 3 months; life expectancy <3 months; the tumor invading the duodenal wall; unstable hematogenic parameters; severe and active infection; and the patient having jaundice owing to biliary obstruction. Men patients are about 1.7 folds more than women; patient's age ranges from 15 to 89 years with a mean value of 60.8 years; cancers in the pancreas head are a little more than those in the body and tail; most patients have TNM-III and IV cancers; cancer size ranges from 2 cm to 11.9 cm with a mean value of 4.76 cm as listed in Table 1. It is important to note that detailed information about patients and cancer is not released in every clinical report.

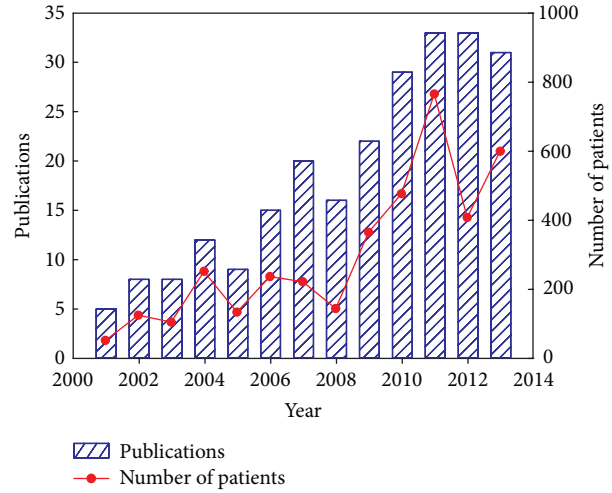


Figure 1: The number of publications and advanced pancreatic cancer patients treated with HIFU or in conjunction with chemotherapy or radiotherapy from 2001 to 2013.

Table 1: Statistics of advanced pancreatic cancer patients treated with HIFU or in conjunction with chemotherapy or radiotherapy.

Men	Women	Age (year) ($n = 3250$)	Head	Body and tail	TNM-II	TNM-III	TNM-IV	Size (cm) ($n = 339$)
2014 (62.98%)	1184 (37.02%)	15–89 Mean: 60.8	1341 (53.68%)	1157 (46.32%)	69 (3.4%)	996 (49.14%)	962 (47.4%)	2–11.9 Mean: 4.76

Table 2: Statistics of the number of sessions, pain relief, clinical benefit rate, and survival of advanced pancreatic cancer patients undergoing HIFU therapy or in conjunction with chemotherapy or radiotherapy.

	Session	Pain relief	Complete relief (CR)	Partial relief (PR)	Clinical beneficial rate (CBR)	Survival (months)
HIFU	6.7 ($n = 653$)	71.33% ($n = 1938$)	29.66% ($n = 1534$)	39.83% ($n = 1534$)	71.06% ($n = 508$)	10.03 ($n = 806$)
HIFU + chemo	7.4 ($n = 471$)	59.72% ($n = 602$)	8.35% ($n = 395$)	45.39% ($n = 395$)	74.76% ($n = 353$)	10.16 ($n = 270$)
Chemotherapy		31.5% ($n = 261$)	4.31% ($n = 100$)	23.22% ($n = 100$)	38.85% ($n = 222$)	7.40 ($n = 112$)
HIFU + radio	5.2 ($n = 130$)	65.91% ($n = 176$)	27.84% ($n = 176$)	38.07% ($n = 176$)	82.15% ($n = 89$)	15.55 ($n = 101$)
Radiotherapy		29.65% ($n = 67$)	3.76% ($n = 67$)	25.89% ($n = 67$)	60.36% ($n = 95$)	

Vital signals, such as respiration and heart rate, blood pressure, and oxygen and carbon dioxide saturation, should be monitored during the HIFU ablation. Anesthesia may be used either to avoid the painful experience or to guarantee immobilization of the target [19]. HIFU is usually carried out as a day case procedure, and average of 6.7 sessions are carried out on patients. Substantial reduction of tumor-related pain can be achieved in most cases even after one HIFU session. Pain relief, including complete relief (CR) and partial relief (PR), is about 71.33% in reported 1938 cases as listed in Table 2. The quality of life, such as appetite, sleeping, and mental status, is improved in most cases and the mean clinical benefit rate (CBR) in 508 cases is 71.06%. The mechanism of pain relief is not fully understood but hypothesized to the following mechanisms: the nerve fibers in the tumor are damaged or undergo apoptosis by the thermal effects; the targeted solar plexus may be inactivated to block the pain signal to be transferred to the brain; the pressure on the nerve applied by the tumor would be reduced due to tumor shrinkage. However, HIFU has less or no effect on the relief of obstructive pain. Average KPS increase by HIFU in reported 290 cases is about 1.5 folds. Survival is evaluated by means of the Kaplan-Meir method. In 806 cases, the median survival is 10.03 months. HIFU can kill tumor cells and block blood supply. Subsequently, potential micrometastases and lymph metastasis can be reduced, but not completely removed.

The presence of scattered intravascular thrombi after HIFU ablation will lead to malabsorption of tissue necrosis and slow atrophy of the cancer fibrosis. Structures around the pancreas determine that HIFU ablation on the pancreatic cancer is mostly palliative in nature and will not conformally reduce it as for the other solid tumors. As a result, the size of ablated tumors may not be significantly reduced but may even be increased in the short term due to the edema on the edge, which is shown in Table 3. Therefore, the pancreatic cancer size cannot be used to evaluate the efficacy of HIFU. In addition, the feasibility of echo in the target varies significantly in the clinical reports, from 0% to 100%. Enhanced echogenicity is mainly due to the presence of cavitation or boiling bubbles, and its size is smaller than the actual size of the thermal lesion.

Vital signs, liver and kidney function, skin burns, local reactions, and systemic effects are monitored and recorded before, during, and after HIFU. All of the side effects associated with HIFU ablation and reported in the published papers are summarized and shown in Figure 2. Most of them are moderate and minor complications, such as first and second degree superficial skin burns, edema, fever, tumor warming, gastrointestinal (GI) dysfunction (e.g., abdominal distension and anorexia with slight nausea), and mild abdominal pain in the treated regions [25]. The subcutaneous layer and skin are occasionally thickened and swollen, and subsequently the echogenicity is increased. Those minor complications are inevitable and mostly associated with ultrasound itself. Pancreatitis is a critical concern because HIFU can mechanically lyse cells and release pancreatic enzymes. However, pancreatic cells do not undergo lysis in thermal fixation until the intracellular enzymes have been completely denatured and inactivated, which minimizes the risk of pancreatitis in HIFU ablation. 15 cases of pancreatitis were acute and recovered usually within a week. In a study of 35 pancreatic cancer patients, all of them had vertebral body necrosis of the anterior half and 10 patients had subcutaneous fat necrosis as identified by MRI (see Figure 3) [7]. All cases were asymptomatic with no need for further treatment. Patients with extrahepatic biliary obstruction were inserted with intestinal

Table 3: Statistics of tumor size change, echogenicity in B-mode ultrasound image, and Karnofsky performance scale (KPS) score of advanced pancreatic cancer patients undergoing HIFU therapy.

Size decrease (n = 629)	Size increase (n = 629)	Echo (n = 186)	KPS % (n = 290)		
			Prior HIFU	Post HIFU	Increase
163 (25.91%)	57 (9.06%)	0%–100% Mean: 73.12%	38.1 ± 17.8~67.8 ± 9.4	74 ± 15~85.71 ± 4.95	151.77%

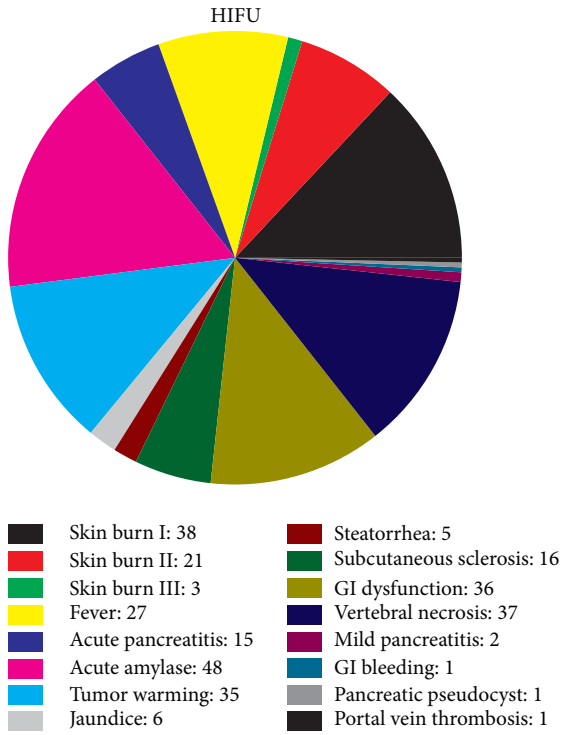


Figure 2: Summary of the complications found in HIFU ablation for advanced pancreatic cancer.

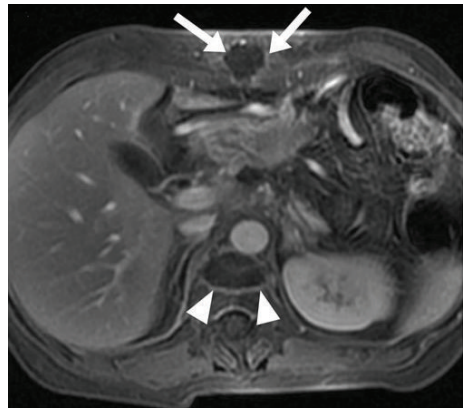


Figure 3: Large rim-enhancing areas of fat necrosis (arrow head) and vertebral body necrosis (arrow) along the ultrasound propagation path in a pancreatic cancer patient two weeks after HIFU ablation in fat-saturated T1-weighted magnetic resonant image after gadolinium infusion (used with permission [7]).

metal stent before HIFU treatment to reduce jaundice; no deformation, displacement, or occlusion happened to the stent after HIFU treatment. One patient had portal vein thrombosis and was hospitalized for 7 days [26]. Further compression on the portal vein by the edematous tumor after HIFU ablation may be the reason of inappropriate blood clotting. A large pseudocyst surrounded by inflammatory changes was found in the mesentery anterior to the pancreas, which may be caused by the delayed perforation of the cyst near the pancreatic tumor due to damage of the cystic wall [25]. Third degree skin burns were found in the early application of HIFU and could be avoided after appropriate use of water balloon and careful examination of the coupling condition [24]. Two cases of mild pancreatitis were also found in the preliminary application [27]. One transient upper GI bleeding was observed due to a nasogastric tube. Two patients had tumor-duodenal fistulas with severe abdominal pain (see Figure 4) [28]. However, most major complications could be avoided through careful treatment planning and monitoring during the procedure. A low energy is preferred if the necrosis production is effective.

The purpose of posttreatment diagnosis is to verify the generation of necrosis in the target and its size. CT can clearly demonstrate the tumor size and shape as shown in Figure 5. But CT is insensitive to fat tissue, unreliable to assess the functionality of tumor's rim, and difficult for hypovascular tumor. Contrast-enhanced CT or multiple detector CT (MDCT) or magnetic resonance imaging (MRI) is also used before and after HIFU to assess necrosis by the absence of vascularity within the tumor, but not its metabolic activity. Iodinated contrast agents are prohibited to those who are allergic to iodine. Dose of contrast used in MRI is less than that of CT. Its slow injection rate may not cause discomfort or allergy to patients. Diagnostic capability becomes better with multiple dynamic scanning since MRI has nonionization as shown in Figure 6. T1-weighted MRI provides a good image contrast anatomy while T2-weighted one is sensitive to tumor coagulation and necrosis liquefaction. Ultrasound color Doppler is also sensitive to the blood flow inside the tumor as shown in Figure 7. Introduction of ultrasound contrast agent (e.g., microbubbles) could enhance the signal-to-noise ratio and diagnosis accuracy. The tumour size is not a reliable benchmark to evaluate HIFU efficacy in treating pancreatic cancer. Persistence of lack of enhancement suggests successful local tumor control. Contrast-enhanced MRI works excellently in the rapid assessment of therapeutic response of ablated tumor. PET or PET-CT is useful for diagnosing and staging of pancreatic cancer and for evaluating the outcome of HIFU treatment. Single-photon emission computed tomography (SPECT) as shown in Figure 8,

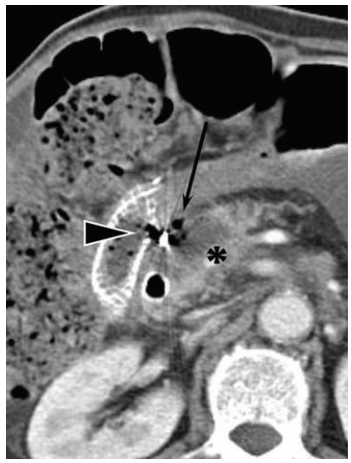


Figure 4: Necrosis (asterisk) in the pancreas head with rim enhancement, a fistula between the pancreatic tumor and the adjacent bowel with the mottled air densities (long thin arrows), and communication between the duodenum and the ablated cavity via focal disruption of the duodenal stent (arrowhead) in a follow-up CT after HIFU ablation (used with permission [7]).

a functional imaging, demonstrates the active metabolism of viable cancer cells. Real-time sonographic imaging is usually performed during the HIFU to examine the echogenicity in both the tumor and acoustic coupling path. However, hyperechoic changes in the target do not precisely correlate with the actual lesion.

In the peripheral region, the tumor cells are lethally damaged. In contrast, those thermally fixed in the central region look normal and similar to viable cells with the preservation of cellular structure as shown in Figure 9. Both electron microscopy and enzyme histochemical examination revealed an irreversible cell death. At autopsy, the tumor was replaced by a scar 10 months after HIFU ablation, and there was no apparent mass lesion remaining [18].

After clinical HIFU treatment, the serum amylase and urinary amylase levels are measured by a radioimmunometric assay as surrogate markers for traumatic pancreatitis. CA19-9, CA242, and CEA can be decreased by 49.41%, 34.93%, and 28.41%, respectively, which demonstrates the absence of pancreatitis as listed in Table 4.

4. Concurrent HIFU with Chemotherapy and Radiotherapy

Chemotherapy and chemoradiation (CRT) is an adjuvant treatment for resected pancreatic cancer but the primary one for locally advanced disease. Adjuvant chemotherapy with 5-fluorouracil or the combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) can increase the 5-year survival (about 10 to 20%) in several large randomized studies. In contrast, adjuvant CRT is controversial with favoring practices in the USA but not recommended in Europe due to the absence of randomized studies. FOLFIRINOX is a new standard for advanced pancreatic cancer but has significant toxicities [29]. Safety data in patients with suboptimal status is not available, so caution should be taken in its use.

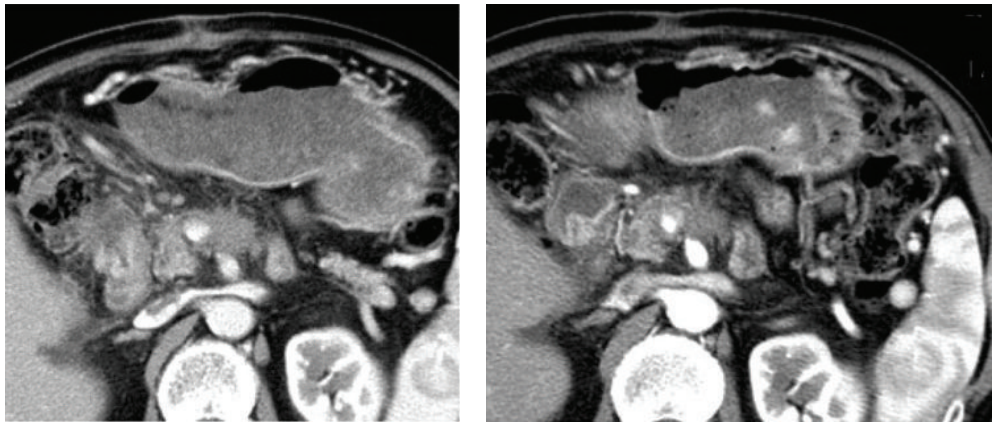
Advanced hypovascular tumors are more sensitive to heat shock due to no vascularity recovery after thermal injury. Thus, combination of HIFU with chemotherapy for advanced tumors is very attractive because the efficacy of chemotherapy is limited by long distance between tumor cells and blood vessels. After chemotherapy, but before HIFU ablation, an increase in blood flow is found inside the tumor using contrast-enhanced ultrasound (CE-US). Then, hypervascularity of the tumor is changed to hypovascularity by HIFU therapy, and the vasculature of large vessels through the tumor remains undamaged. As HIFU increases, the permeability of the vascular endothelial cells (maybe due to both intravascular cavitation and thermal effects) allows the chemotherapeutic agent to penetrate through the vessel into the interstitial space of the tumor, aids the distribution of the chemotherapeutic agent (pharmacokinetics) into the tumor due to the acoustic radiation force, and inhibits tumor cells to repair damage to chemotherapy [30]. Reduction of the vascularity through the tumor delays the drug clearance and increases the drug concentration. So ultrasound hyperthermia can reduce the dosage required and adverse effects of chemotherapy. Although the working principle of various chemotherapeutic agents and their targeted stage on cell metabolism and proliferation are different, combined HIFU and chemotherapy all result in a better outcome, high pain relief, CBR, and longer survival. In addition, intra-artery instead of vein injection can reduce the concentration in the circulating system and enhance the tolerability of patients.

HIFU ablation followed by radiotherapy is also a promising method. Reduced blood flow can prevent heat dissipation, lead to tumor cell damage and hypoxia, increase the cytotoxicity, and improve the sensitivity of radiotherapy. Radiotherapy is effective for oxygen-rich cells, and hyperthermia, in contrast, works well for hypoxic ones. Since fibrosis produced after hyperthermia influences radiation effects, HIFU is carried out after or simultaneously with the radiotherapy.

Clinical studies show that a combination of HIFU and chemotherapy or radiotherapy can achieve a higher CBR and longer survival than the single modality. The observed side effects are associated with HIFU, chemotherapy, and radiotherapy themselves. There is no enhancement in the complications by combining therapeutic modalities as shown in Figure 10. Chinese herbals were also used in conjunction with HIFU treatment [31, 32]. However, the number of cases is too small to fully evaluate its efficacy.

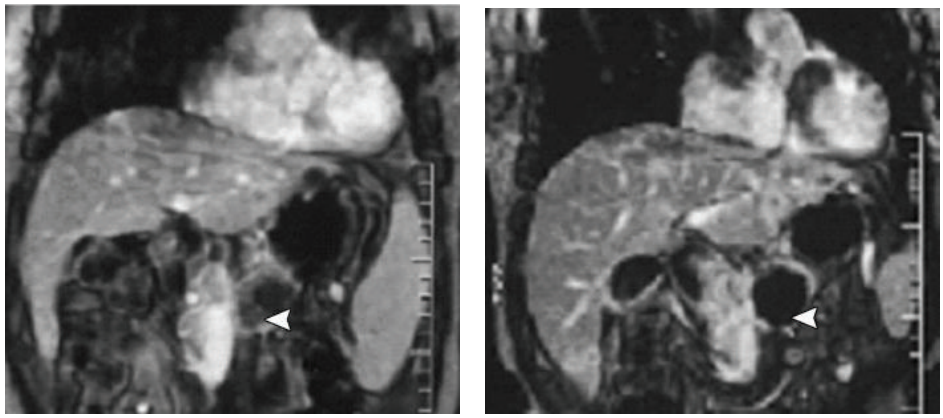
5. HIFU-Induced Immune Response

Recently, HIFU-induced immune response, suppression of the activity of tumor, and downregulation of tumor markers have attracted attention as an effective approach of cancer treatment. Selective recognition and destruction of tumor cells by the host immune system play an important role in antitumor immunity, which requires expression of tumor antigens. The immune system in most cancer patients fails



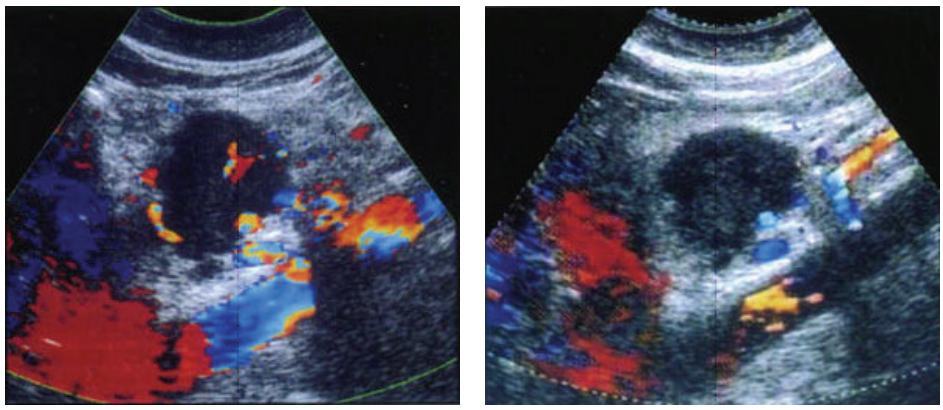
(a) (b)

Figure 5: CT imaging shows no apparent change of pancreas (a) before and (b) after HIFU therapy (used with permission [18]).



(a) (b)

Figure 6: Dynamic contrast-enhanced gradient-echo T1-weighted MR images (a) before and (b) 2 weeks after HIFU ablation for advanced pancreatic cancer with a diameter of 4.5 cm. No evidence of contrast enhancement in the treated lesion (arrowhead) illustrates complete coagulation necrosis (used with permission [19]).

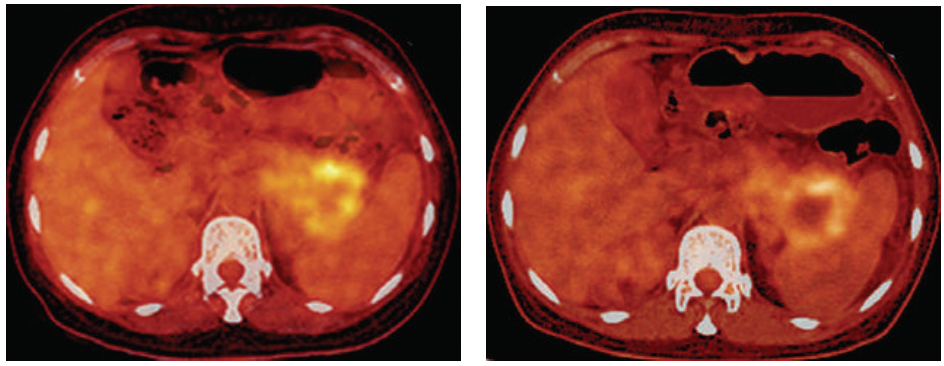


(a) (b)

Figure 7: Sonography of pancreatic cancer (a) before and (b) after HIFU therapy showing the enhancement of echogenicity in the tumor but decrease of vascularity, an indicator of coagulative necrosis (used with permission [20]).

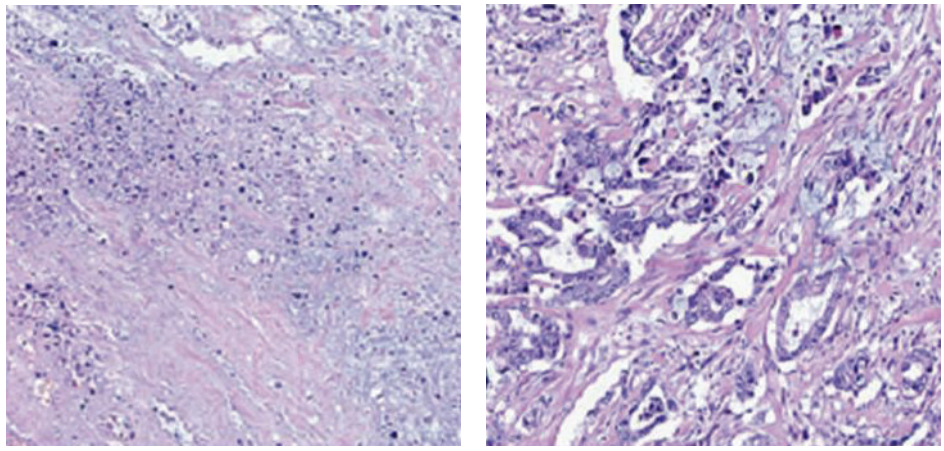
Table 4: Comparison of the serum levels before and after HIFU treatment.

	CA19-9 (U/mL)		CA242 (U/mL)		CEA (ng/mL)	
	Pre-HIFU	Post-HIFU	Pre-HIFU	Post-HIFU	Pre-HIFU	Post-HIFU
Range	42.6 ± 8.6~583.8 ± 20.4	21.5 ± 6.6~305.7 ± 19.3	73.6 ± 41.7~114.4 ± 42.0	46.3 ± 13.4~85.2 ± 21.9	38.4 ± 12.4~53.8 ± 17.3	18.9 ± 33~33.9 ± 14.8
Decrease	49.41% (n = 701)		34.93% (n = 135)		28.41% (n = 114)	



(a) (b)

Figure 8: A PET-CT scan made (a) before HIFU demonstrates a SUVmax of 7.5 g/mL and (b) 3 months after HIFU demonstrates coagulative necrosis inside the tumor and the decreased SUVmax of 5.3 g/mL (used with permission [21]).



(a) (b)

Figure 9: H&E staining of pancreatic cancer after HIFU ablation shows (a) disappearance of nuclei and necrosis and (b) the thermally fixed cancer cells (used with permission [22]).

to control the development and growth of initial cancer and to prevent local recurrence and metastasis after conventional therapies due to poor tumor antigen processing and immune-suppressive cytokines released by the tumor. HIFU can activate a host antitumor immunity to control micrometastasis and generate tumor resistance [33]. Increased NK cell activity, the population of CD4+ lymphocytes, and the ratio of CD4+/CD8+ in the blood circulation of cancer patients are found after HIFU ablation as listed in Table 5. Some clinical studies have shown greater concentrations of dendritic cells, macrophages, and B lymphocytes in the HIFU treatment group. Till now, the underlying mechanisms of antitumor immunity enhancement are not completely understood. Large amounts of tumor debris produced by HIFU can be released and reabsorbed in situ. A variety of tumor antigens remain in the tumor debris with and without typical characteristics of thermal damage. Circulating T cells activate specifically toward tumor antigens. High temperature unfolds the proteins from the native state to a more random state of lower organization, which can lead to either loss or preservation of antigenic determinants. Upregulation of heat shock protein (HSP) by hyperthermia can also stimulate the immune response. HIFU destructs the tumor structure and lowers its viability as well as the suppression of the immune system. Aseptic inflammation induced by pancreatic necrosis in HIFU ablation leads to the local accumulation of IL-1 and

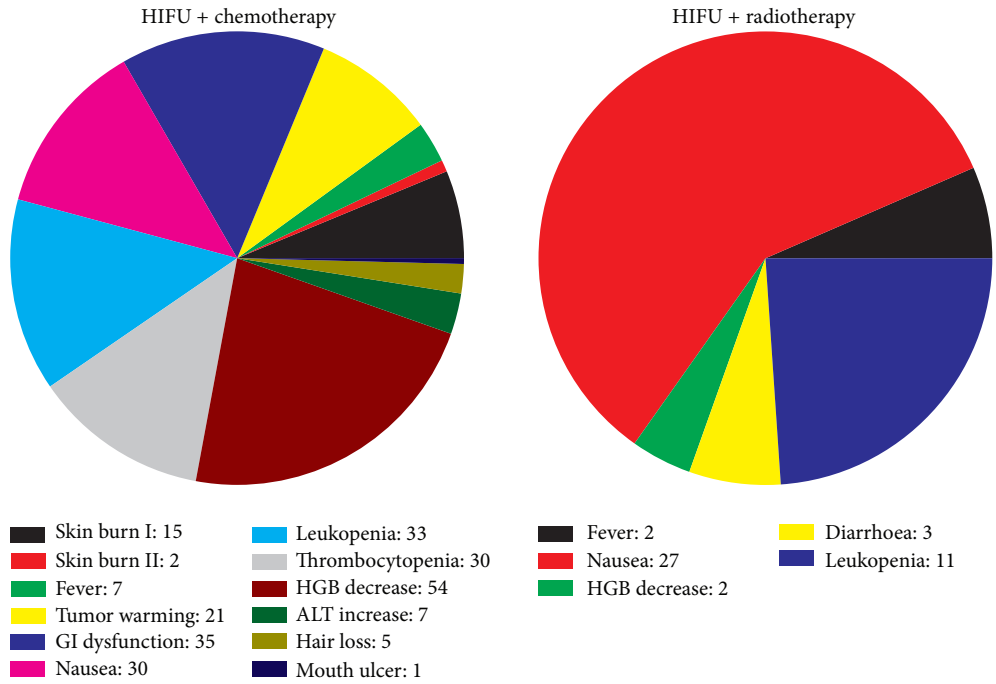


Figure 10: Summary of the complications found in the combination of HIFU ablation with chemotherapy and radiotherapy for advanced pancreatic cancer.

Table 5: Statistical summary of immune factors before and after HIFU ablation in pancreatic cancer patients.

	Pre-HIFU	Post-HIFU	Increase
CD3+ (n = 141)	37.39 ± 11.78~60.3 ± 5.9	51.8 ± 6.4~59.6 ± 6.7	112.94%
CD4+ (n = 93)	24.19 ± 7.02~32.6 ± 5.4	28 ± 10~34.7 ± 5.3	108.89%
CD4+/CD8+ (n = 93)	0.9 ± 0.3~1.1 ± 0.1	1.09 ± 0.53~1.4 ± 0.1	125.9%
NK (n = 28)	20.54 ± 9.1~21 ± 9	25 ± 13~25.52 ± 11.9	121.8%

IL-2, which would adjust the antitumor immunity of host [34, 35].

6. Comments for Better Outcome

Although HIFU is an overall safe and noninvasive therapeutic modality for pancreatic cancer, it requires careful preoperative preparation as well as operative performance [36]. Understanding the factors for complications, recruiting appropriate patients, preparing the preoperation carefully, selecting proper HIFU operation parameters, and paying attention to adjacent vital organs during the procedure are necessary steps for minimizing severe complications [7]. Patients with extensive scars or scars lying in the path of the acoustic beam should be excluded because scar tissue absorbs ultrasound strongly and may result in a skin burn. Obstruction of bowel gas or bone to acoustic wave propagation towards the target should be removed to minimize the risk of causing unintended thermal injury. Therefore, the gas in the stomach and colon should be evacuated by careful bowel preparation, such as liquid food and no milk for 3 days, fasting for 12 hours before treatment, an enema in the early morning on the day of treatment, insertion of a urinary catheter (catharsis), and intraoperative bladders pressure. Drinking degassed water can remove the bowel gas quickly, but it has a short effective duration. Medicine may be more helpful, such as oral administration of quick-solution gastroenter-ultrasound developer. The skin at the wave entry site would be shaved to avoid the trapping of bubbles, degassed with a vacuum cup aspiration device, and degreased with 95% alcohol. Artificial pleural effusion may be placed if necessary to ensure the acoustic window. Proper positioning is selected by observing the acoustic path in the sonography. Applying slight abdominal pressure to the abdomen, such as using a soft water balloon, also helps to compress the bowel and clear the acoustic window. Respiratory motion during the treatment spreads the acoustic energy over a larger area in the target than expected and may result in incomplete tumor coagulation and damage to adjacent tissues. If it is too serious in operation, general anesthesia with endotracheal intubation and mechanical ventilation would be applied to allow provisional suspension of breath with controlled pulmonary inflation as well as reduction of pain and discomfort associated with HIFU ablation. Tracking the respiratory motion in real time would allow for rapid focus

shifting in sync with the target position but needs proof in practice. Operators must monitor the imaging changes on adjacent vital organs, such as the myocardium, diaphragm, and bowel loops. Detection of the complications as early as possible allows the provision of appropriate and immediate management. A large-aperture transducer could decrease the acoustic intensity at the body surface and reduce the propensity of skin burn because of a wide convergent angle, which is a hypothesis that needs more clinical or *in vivo* evidence. If tumors are located in the pancreatic head, there remains a substantial possibility of biliary obstruction or biliary duct damage caused by the thermal ablation. An endobiliary stent should be routinely placed before HIFU ablation. At high power, each session should be within 1 hour. Lesions should cover the whole tumor area, and multiple sessions will be performed for satisfactory long-term outcome.

7. HIFU Challenges

Despite the large number of clinical cases of HIFU on advanced pancreatic cancer with promise, large-scale randomized and controlled trials at multiple centers with long-term follow-up have not been carried out to date to confirm these findings or to determine whether HIFU can improve overall survival by inducing local tumor response with or without chemotherapy, radiotherapy, or targeted drug [37]. Experiences in China may not be applicable to the Western countries. Appropriate HIFU treatment planning for complete coagulation but sufficient tissue margin is desirable in order to reduce the recurrence. Standard criteria are also required to evaluate both the short- and long-term efficiency and efficacy of HIFU on advanced pancreatic cancer. Pretreatment of HIFU on the margin of resectable pancreatic tumor may also be good for the better surgical outcome. A standardized dose of HIFU, chemotherapy, or radiotherapy has not been established, so current use is mostly empirical. An effective combination of treatment modalities is currently under investigation.

It seems clear that HIFU is finding its roles in clinics, although its technical development is still in its infancy [16]. Future developments will involve speeding up treatments and improving treatment targeting and monitoring. Motion artifact due to respiration and heartbeat is also a concern in clinics and needs to be monitored in real time for consistent delivery of HIFU energy during either end expiration or inspiration. An alternative solution is electrically steering the focus by the phased-array in order to keep the exposed target consistently. Ideally, the tissue inhomogeneity and attenuation can be compensated using phased-array design for accurate beam forming. In order to estimate the thermal dose, the acoustic output of the device, the acoustic and biological characteristics of the tumor, and the attenuation along the ultrasound pathway (primarily abdominal wall and viscera) are required [23]. One of the major factors that limit the wide application of HIFU is the absence of ultrasound-based thermometry and low frame rate and resolution of MRI-based one. HIFU system needs to be improved to work more appropriately for advanced pancreatic cancers.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Mast Cells Density Positive to Tryptase Correlates with Angiogenesis in Pancreatic Ductal Adenocarcinoma Patients Having Undergone Surgery

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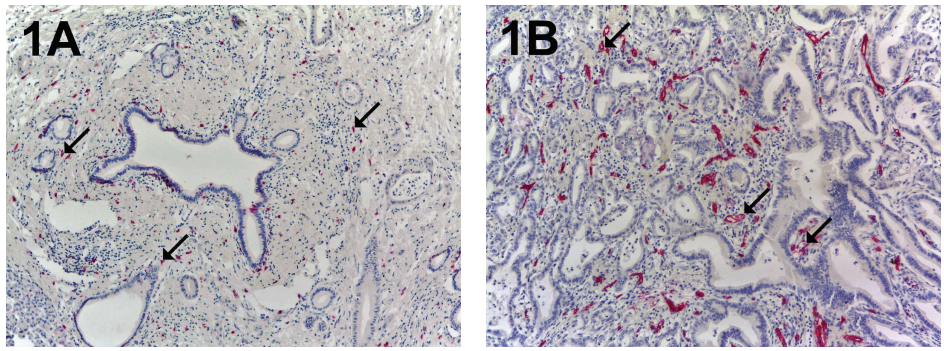
Background. Literature data suggest that cells such as mast cells (MCs), are involved in angiogenesis. MCs can stimulate angiogenesis by releasing of several proangiogenic cytokines stored in their cytoplasm. In particular MCs can release tryptase, a potent *in vivo* and *in vitro* proangiogenic factor. Nevertheless few data are available concerning the role of MCs positive to tryptase in primary pancreatic cancer angiogenesis. This study analyzed MCs and angiogenesis in primary tumour tissue from patients affected by pancreatic ductal adenocarcinoma (PDAC). *Method*. A series of 31 PDAC patients with stage $T_{2\text{-}3}N_{0\text{-}1}M_0$ (by AJCC for Pancreas Cancer Staging 7th Edition) was selected and then underwent surgery. Tumour tissue samples were evaluated by means of immunohistochemistry and image analysis methods in terms of number of MCs positive to tryptase (MCDPT), area occupied by MCs positive to tryptase (MCAPT), microvascular density (MVD), and endothelial area (EA). The above parameters were related to each other and to the main clinicopathological features. *Results*. A significant correlation between MCDPT, MCAPT, MVD, and EA group was found by Pearson's t-test analysis (r ranged from 0.69 to 0.81; P value ranged from 0.001 to 0.003). No other significant correlation was found. *Conclusion*. Our pilot data suggest that MCs positive to tryptase may play a role in PDAC angiogenesis and they could be further evaluated as a novel tumour biomarker and as a target of antiangiogenic therapy.

1. Introduction

Inflammatory cells, such as macrophages, lymphocytes, and mast cells (MCs), play a major role in tumour angiogenesis by means of angiogenic cytokines stored in their cytoplasm. MCs are involved in neovascularization in experimentally induced tumour, accumulate near to tumour cells before the angiogenesis onset, and participate in the metastatic spreading of primary tumours. MCs intervene in angiogenic process releasing classical proangiogenic factors, such as vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), fibroblast growth factor-2 (FGF-2), and the nonclassical proangiogenic factor, namely, tryptase stored in their secretory granules [1–9]. The role of MCs has been



(a) (b)

Figure 1: In (a) a pancreatic ductal adenocarcinoma sample stained with the anti-tryptase antibody. Many scattered red immunostained MCs. Arrows indicate single MC. Magnification: in (b) a highly vascularized pancreatic ductal adenocarcinoma sample stained with the anti-CD-31 antibody. Many red immunostained microvessels. Arrows indicate microvessel. Magnification: (a-b), ×100.

broadly studied in benign lesions, in animal and human's cancers, such as keloids, mast cells tumours, and head and neck, colorectal, gastric, lung, and cutaneous malignancies, indicating that MCs density is highly correlated with the extent of tumour angiogenesis [10–14]. Recent data have shown that MCs density is correlated with angiogenesis and progression of patients with pancreatic cancer [15, 16]. However, no data have been published regarding the correlation each to other of MCs density positive to tryptase (MCDPT), area occupied by MCs positive to tryptase (MCAPT), microvascular density (MVD), endothelial area (EA) and the main clinicopathological features in primary tumour tissue of affected patients. To this end, we conducted a prospective study in a series of 31 pancreatic ductal adenocarcinoma patients (PDACP) having undergone surgery with stage $T_{2\text{-}3}N_{0\text{-}1}M_0$ (by AJCC for Pancreas Cancer Staging 7th Edition). Tumour tissue samples were evaluated by means of immunohistochemistry and image analysis methods, obtaining a significant correlation between MCDPT, MCAPT, MVD, and EA group. Our pilot data suggest that MCs positive to tryptase may play a role in PDAC angiogenesis and they could be further evaluated as a novel tumour biomarker and as a target of antiangiogenic therapy.

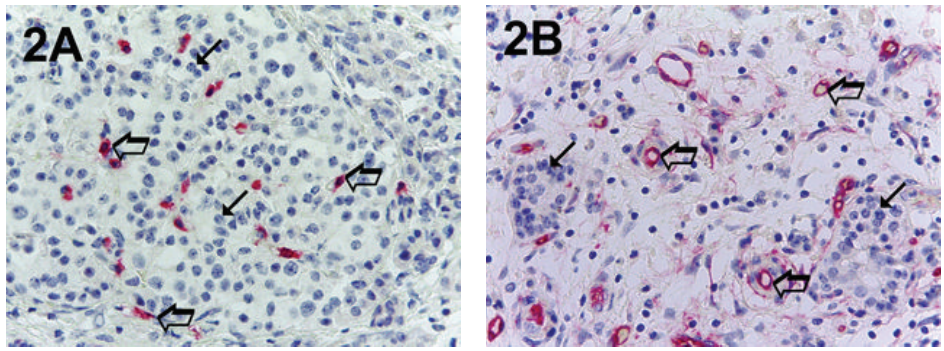
Table 1: Clinicopathological features of patients.

	N
Overall series	31
Age	
(i) <65	23
(ii) >65	8
Sex	
(i) Male	25
(ii) Female	6
Tumour site	
(i) Head	13
(ii) Body-Tail	18
TNM by AJCC for Pancreas Cancer Staging 7th Edition	
(i) $T_2N_{0\text{-}1}M_0$	14
(ii) $T_3N_{0\text{-}1}M_0$	17
Histologic type	
Ductal adenocarcinomas	31
Histologic grade	
(i) G1-G2	19
(ii) G3	12

2. Patients and Methods

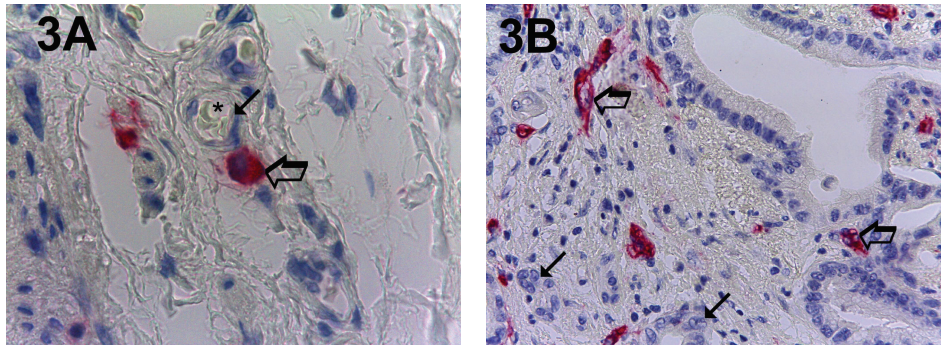
2.1. Patients. The clinicopathological features of selected patients are summarized in Table 1. A total of 31 PDACP patients underwent potential curative resection. Surgical approaches used were pancreaticoduodenectomy, distal pancreatectomy, and total pancreatectomy with lymph node dissection. Patients were staged according to the American Joint Committee on Cancer 7th Edition (AJCC-TNM) classification and the World Health Organization classification (2000 version) was used for pathologic grading. All patients had no distant metastases on computed tomography and ten patients had received neoadjuvant-therapy based on Gemcitabine or FOLFIRINOX. The study was approved by the Ethics Committee of "Mater Domini" Hospital, "Magna Graecia" University, Catanzaro, and from each enrolled patient the signed informed consent was obtained.

2.2. Immunohistochemistry. For the evaluation of MCDPT, MCAPT, MVD, and EA, a three-layer biotin-avidin-peroxidase system was utilized [17]. Briefly, 4 μm thick serial sections of formalin-fixed and paraffin-embedded surgically removed tumour samples were deparaffinised. Then, for antigen retrieval, sections were microwaved at 500 W for 10 min, after which endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Next, adjacent slides were incubated with the monoclonal antibodies anti-CD31 (clone JC70a; Dako) diluted 1 : 40 for 30 min at room temperature and anti-tryptase (clone AA1; Dako, Glostrup, Denmark) diluted 1 : 100 for 1 h at room temperature.



(a) (b)

Figure 2: In (a) pancreatic ductal adenocarcinoma sample stained with the anti-tryptase antibody. Many scattered red immunostained MCs. Big arrows indicate single red MC and small arrows indicate the bleu nucleus of cancer cells. In (b) a highly vascularized pancreatic ductal adenocarcinoma sample stained with the anti-CD-31 antibody. Big arrows indicate single red microvessels with a lumen and small arrows indicate the bleu nucleus of cancer cells. Magnification: (a-b), ×400.



(a) (b)

Figure 3: In (a) pancreatic ductal adenocarcinoma sample stained with the anti-tryptase antibody. Big arrow indicates a single red MC and small arrow indicates a microvessel with its lumen. The lumen is marked with an asterisk and there are well visible intraluminal red blood cells. In (b) a highly vascularized pancreatic ductal adenocarcinoma sample stained with the anti-CD-31 antibody. Big arrows indicate single red microvessels with their own lumen and small arrows indicate the bleu nucleus of cancer cells. Magnification: (a-b), ×1000 in oil.

The bound antibody was visualised using biotinylated secondary antibody, avidin-biotin peroxidase complex, and fast red. Nuclear counterstaining was performed with Gill's haematoxylin number 2 (Polysciences, Warrington, PA, USA). Primary antibody was omitted in negative controls.

2.3. Morphometric Assay. An image analysis system (Semiquantimet 400 Nikon) was employed.

The five most vascularized areas ("hot spots") were selected at low magnification and both MCDPT (Figure 1(a)) and individual vessel (Figure 1(b)) were counted at ×400 magnification (0.19 mm^2 area; Figures 2(a) and 2(b)) (GR and NZ) [1]. Single red stained endothelial cells, endothelial cell clusters and microvessels, clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements were counted [17]. Areas of necrosis were not considered for counting. In serial sections each single MC positive to tryptase was counted. Single red stained endothelial cells and red MCs positive to tryptase were also evaluated in terms of immunostained area at ×400 magnification (0.19 mm^2 area) [17]. Finally morphological detail of both MCs positive to tryptase and endothelial cells was observed at ×1000 magnification in oil (Figures 3(a) and 3(b)).

2.4. Statistical Analysis. Linear correlations between MCDPT, MCAPT, MVD, and EA groups were quantified by means of Pearson's correlation coefficient (r). Correlation between MCDPT, MCAPT, MVD, and EA groups and the main clinicopathological features were analysed by chi-square test. In all analyses a $P < 0.05$ was considered significant. All statistical analyses were performed with the SPSS statistical software package (SPSS, Inc., Chicago, IL).

3. Results

Immunohistochemical staining by using the antibodies anti-CD31 and anti-tryptase allows demonstration of that in highly vascularized cancer tissue; MCs positive to tryptase are well recognizable and generally they are located in perivascular position (Figure 3(a)).

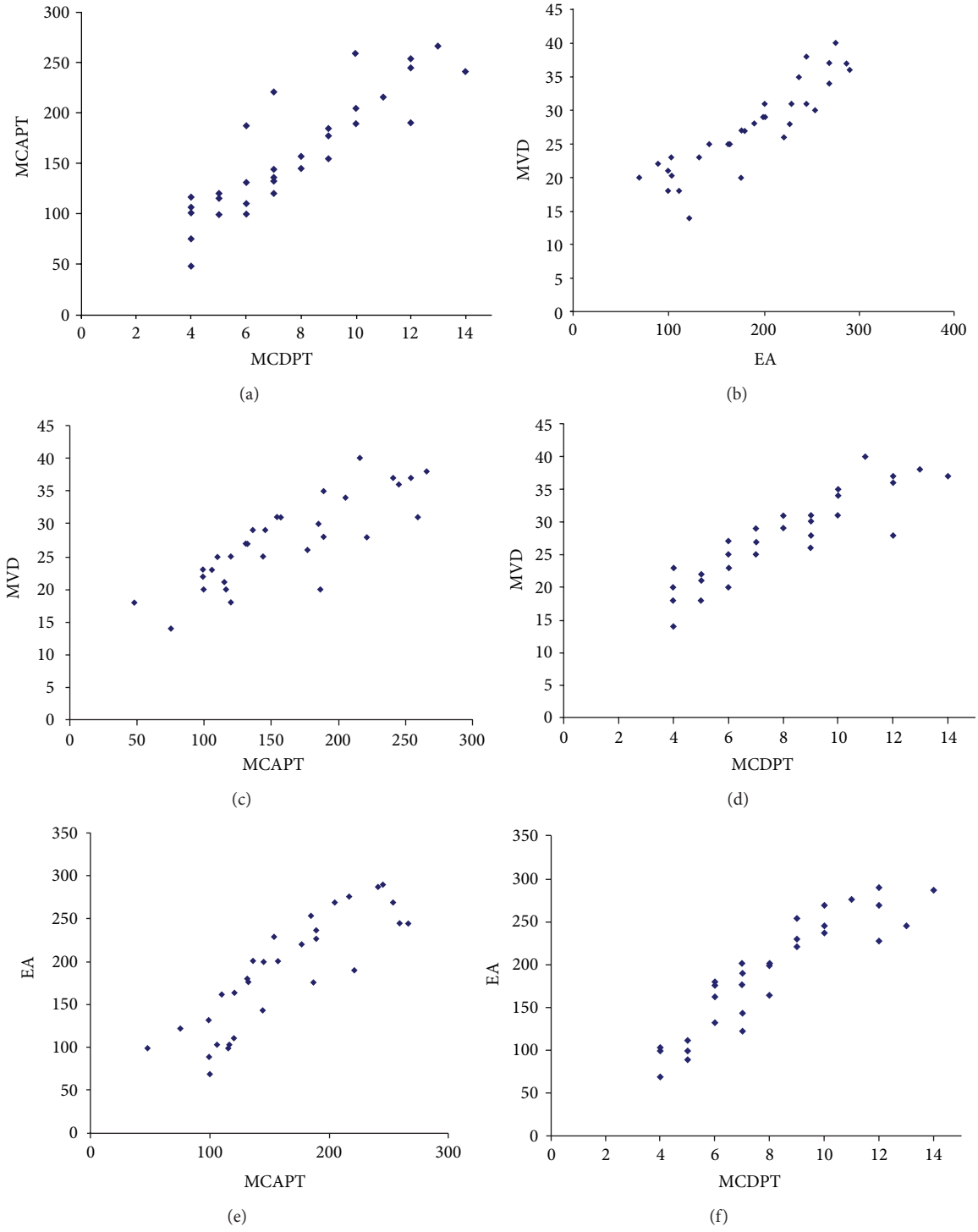


Figure 4: Correlation analysis between MCDPT and MVD ($r = 0.81$; $P = 0.001$), MCAPT and MVD ($r = 0.69$; $P = 0.003$), MCDPT and EA ($r = 0.76$; $P = 0.002$), MCAPT and EA ($r = 0.73$; $P = 0.002$), MVD and EA ($r = 0.80$; $P = 0.001$), and MCDPT and MCAPT ($r = 0.77$; $P = 0.001$).

Mean values ± 1 SD of all the tissue evaluated parameters are reported in Table 2. There was a significant correlation between MCDPT and MVD ($r = 0.81$; $P = 0.001$), between MCAPT and MVD ($r = 0.69$; $P = 0.003$), between MCDPT and EA ($r = 0.76$; $P = 0.002$), between MCAPT and EA ($r = 0.73$; $P = 0.002$), between MVD and EA ($r = 0.80$; $P = 0.001$), and between MCDPT and MCAPT ($r = 0.77$; $P = 0.001$) (Figure 4). No correlation concerning MCDPT, MCAPT, MVD, EA, and the main clinicopathological features was found.

Table 2: MCAPT, MCDPT, EA, and MVD means ± 1 standard deviations.

MCDPT ×400 magnification (0.19 mm^2 area)	MCAPT ×400 magnification (0.19 mm^2 area)	EA ×400 magnification (0.19 mm^2 area)	MVD ×400 magnification (0.19 mm^2 area)
8 ± 3[a]	159.38μ^{2a} ± 58.30[a]	186.06μ^{2a} ± 65.89	27 ± 8[a]

[a]Mean ± 1 standard deviation.

4. Discussion

MCs' involvement in tumour angiogenesis has been demonstrated in several animals models and human malignancies [10–14, 18–20].

MCs are recruited and activated via several factors secreted by tumour cells, such as the C-Kit receptor or stem cells factor, VEGF, FGF-2, and TP. In tumour microenvironment, MCs secrete both gelatinases A and B which, in turn, degrade extracellular matrix, releasing stored angiogenic factors [21–33].

On the other hand, MCs may induce angiogenesis by several proangiogenic factors stored in their secretory granules, such as VEGF, FGF-2, tumour necrosis factor alpha, and interleukin 8, transforming growth factor beta, heparin, and tryptase. With special reference to the last, it is involved in tumour angiogenesis stimulating the formation of vascular tubes in *in vitro* and *in vivo* experimental models and it is also an agonist of the PAR-2 in vascular endothelial cells that, in turn, induces angiogenesis. Interestingly in several human malignancies but not in pancreatic cancer, MCDPT and MCAPT have been associated with tumour angiogenesis. In this regard experimental results suggested that MCDPT may stimulate pancreatic cancer cells contributing to pancreatic tumour progression [34–40].

Published data from Esposito et al. [41] showed that mononuclear inflammatory cells of the nonspecific immune response are recruited in pancreatic cancer tissues and they are able to stimulate angiogenesis and cancer progression.

In this pilot study, we have evaluated the correlations between MCDPT, MCAPT, MVD, and EA in a series of 31 PDACP having undergone surgery and our results suggest an association between tryptase and microvascular bed. We found this correlation in double way: first in terms of number of positive tryptase cells and immunostained microvessels and second in terms of extension of positive tryptase area and immunostained microvessels area. To avoid methodological bias the evaluation of MCDPT, MCAPT, MVD, and EA has been performed by means of an image analysis system at ×400 magnifications in a well-defined microscopic area of 0.19 mm^2 as previously published in other tumours types [1]. Our preliminary data agree on the biological role of tryptase as a strong proangiogenic factor. In this manner we suggest that tryptase from MCs may play a role also in pancreatic tumour tissue angiogenesis. Further studyin a large series of patients will be necessary to confirm our first results. In this context, the evaluation of MCs positive to tryptase may be a novel surrogate angiogenic marker in pancreatic cancer able to predict angiogenic index. We hypothesize also to stop pancreatic angiogenesis inhibiting mast cell degranulation by means of C-Kit inhibitors or targeting tryptase by means of gabexate mesilate or nafamostat mesilate [42–45]. Further studies in more large series of patients are awaited regarding this very intriguing topic.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Aberrant MicroRNAs in Pancreatic Cancer: Researches and Clinical Implications

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with a high rate of mortality and poor prognosis. Numerous studies have proved that microRNA (miRNA) may play a vital role in a wide range of malignancies, including PDAC, and dysregulated miRNAs, including circulating miRNAs, are associated with PDAC proliferation, invasion, chemosensitivity, and radiosensitivity, as well as prognosis. Greater understanding of the roles of miRNAs in PDAC could provide insights into this disease and identify potential diagnostic markers and therapeutic targets. The current review focuses on recent advances with respect to the roles of miRNAs in PDAC and their practical value.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is highly malignant and has a poor prognosis. The overall 5-year survival rate for PDAC is less than 5% [1]. Researchers estimated that about 45,220 cases of PDAC were newly diagnosed and 38,460 PC-related deaths occurred in the United States in 2013 [2]. Surgery remains the best choice for PDAC treatment. However, most patients are diagnosed at an advanced stage, making them poor candidates for surgical resection. Lack of early alarming symptoms, rapid local or distant metastasis, highly malignant phenotypes, and innate resistance to conventional chemotherapeutics are the major reasons for the dismal prognosis for PDAC. Therefore, there is an urgent need to develop new diagnostic strategies and prognostic markers as well as potential therapeutic targets to improve the outcome of PDAC patients.

Since first discovered in *Caenorhabditis elegans* in 1993, microRNAs (miRNAs) have unraveled new mechanisms for regulation of gene expression and have provided new directions for cancer research. miRNAs are comprised of a class of highly conserved short noncoding, 17–25 nucleotide long RNA products [3] that regulate gene expression at the posttranscriptional level. They are negative regulators of gene expression through base pair interactions with the 3′ untranslated region (3′UTR) of protein-coding mRNAs. Partial complementarity between the miRNAs and the 3′UTR of the target transcripts leads to inhibition of translation, while perfect complementarity results in degradation of mRNAs [4]. miRNAs are predicted to regulate the activity or gene expression of over 30% of all protein-coding genes in mammals. So far, more than 1800 human miRNAs have been identified [5–8].

Since the discovery of miRNA's involvement in chronic lymphocytic leukemia [9], tremendous studies have validated the fact that aberrant expression of miRNAs is associated with cancers [10–14]. Extensive mapping of miRNA genes showed that these oncomiRs are often located at genomic regions associated with cancer. Previous studies have reported that while elevated expression of some miRNAs is associated with carcinogenesis (oncogenes), others may inhibit cancer by reducing cell proliferation, survival, and cellular differentiation [15]. miRNA expression profiling signatures can distinguish cancer from benign tissues and this may provide the basis for developing new diagnostic and therapeutic strategies [16].

The number of studies on miRNAs in a PDAC setting is increasing at an exponential rate in recent years. However, the potential clinical use of miRNAs in diagnosis and treatment of PDAC and their prognostic value have not been well summarized yet. The present review focuses on recent advances of miRNA research in PDAC and their potential practical value.

Table 1: Important miRNAs deregulated in pancreatic ductal adenocarcinoma.

miRNA	Expression status	Target genes	Potential clinical value*	Reference
miR-21	Upregulation	PTEN, PDCD4, TPM1, TIMP3	D, P, S, T	[22–27]
miR-221/222	Upregulation	CDKN1B (p27), PUMA, PTEN	D, P, T	[22, 23, 28–30]
miR-155	Upregulation	TP53INP1, SEL1L	D, P	[22–24, 31, 32]
miR-196a	Upregulation	HOXB8, ANXA1, HMGA2	D, P	[22, 23, 33–36]
miR-424-5p	Upregulation	SOCS6	P	[28, 37]
miR-10a	Upregulation	HOXA1	P, T	[38]
miR-373	Upregulation	TP53INP1, LATS2, CD44	D	[39]
miR-27a	Upregulation	Spry2	P, T	[40]
miR-210	Upregulation	HOXA1, FGFRL1, HOXA9	P	[24, 32, 33, 41, 42]
miR-15b	Upregulation	CCNE1	P	[28, 34]
miR-181	Upregulation	TIMP3, TCL1	C	[22, 23]
miR-148a, b	Downregulation	DNMT3b, Mtif, CCKBR, BCL2	D	[22, 43, 44]
miR-198	Downregulation	MSLN, PBX-1, VCP	P, T	[45]
miR-146a	Downregulation	TRAF6, IRAK1, Stat1	T	[46]
miR-20a	Downregulation	Stat3	T	[47]
miR-96	Downregulation	KRAS	T	[48]
miR-375	Downregulation	PDK1, 14-3-3zeta	D	[22, 33]
miR-200c	Downregulation	MUC4, MUC16	P, C, T	[49, 50]
Let-7	Downregulation	KRAS, MAPK	T	[51]

*D: biomarker for diagnosis, P: predictive value for prognosis, C: indicator for chemosensitivity, T: potential target for treatment.
PTEN: phosphatase and tensin homolog, PDCD4: programed cell death 4, TPM1: tropomyosin 1, TIMP3: tissue inhibitor of metalloproteinases 3, CDKN1B (p27): cyclin-dependent kinase inhibitor 1B, PUMA: p53 upregulated modulator of apoptosis, TP53INP1: tumor protein 53-induced nuclear protein 1, SEL1L: Sel-1-like, HOXB8: Homeobox B8, ANXA1: annexin A1, HMGA2: high-mobility group AT-hook 2, SOCS6: cytokine-induced signaling 6, LATS2: large tumour suppressor homolog 2, Spry2: Sprouty2, HOXA1: Homeobox A1, FGFRL1: fibroblast growth factor receptor-like 1, HOXA9: Homeobox A9, CCNE1: cyclin E1, TCL1: T cell leukemia/lymphoma 1, DNMT3b: DNA methyltransferase 3b, Mitf: microphthalmia associated transcription factor, CCKBR: cholecystokinin-B receptor, BCL2: B cell lymphoma 2, MSLN: mesothelin, PBX-1: Pre-B-cell leukemia homeobox factor 1, VCP: valosin-containing protein, TRAF6: TNF receptor-associated factor 6, IRAK1: interleukin-1 receptor-associated kinase 1, Stat1: signal transducer and activator of transcription 1, Stat3: signal transducer and activator of transcription 3, PDK1: 3-phosphoinositide dependent protein kinase-1, MAPK: Mitogen-Activated Protein Kinase.

2. Aberrant miRNA Expression Patterns in PDAC

In recent years, numerous approaches have been developed to quantify miRNA levels [17–19]. These approaches have identified distinct cell- and tissue-specific miRNA expression patterns in PDAC specimens as compared with controls (Table 1). The earliest report regarding pancreas showed that miR-375 and miR-376 were expressed at higher levels in mouse pancreas and pancreatic islet cells than in mouse brain, heart, and liver tissues [20]. Following studies showed that the expression of miR-376 precursor in PDAC cell line PANC-1 was among the highest of all cell lines studied, while expression of miR-375 in the two PDAC cell lines studied did not differ from the other cell lines [21].

Accumulating efforts were then made to explore the miRNA expression signature that is associated with PDAC. Employing RT-PCR, Eun et al. profiled more than 200 miRNA precursors in specimens of human PDAC, paired benign tissue, and normal pancreas. One hundred miRNA precursors were aberrantly expressed in PDAC or desmoplasia, including miRNAs that were previously reported in other human cancers, for example, miR-21, miR-155, miR-221, miR-222, and miR-424-5p, as well as those that were not previously reported in cancers, for example, miR-376a and miR-301. Most of the top aberrantly expressed miRNAs displayed increased expression in tumors. Reverse transcription in situ PCR showed that three of the top differentially expressed miRNAs (miR-221, miR-376a, and miR-301) were localized in tumor cells but not in stroma, normal acini, or ducts [28]. In another study, Bloomston et al. compared the global miRNA expression pattern of resected pancreatic cancer with matched benign adjacent pancreatic tissue and chronic pancreatitis. Specimens were obtained from microdissected paraffin blocks. The miRNA microarray result demonstrated that twenty-one miRNAs with increased expression and 4 with decreased expression were identified and correctly differentiated pancreatic cancer from benign pancreatic tissue in 90% of samples by cross-validation. Fifteen overexpressed and 8 underexpressed miRNAs differentiated pancreatic cancer from chronic pancreatitis with 93% accuracy. Upregulation of miR-155, miR-181a,b,c,d, miR-21, miR-196a, and miR-221 and downregulation of miR-148a,b and miR-375 could differentiate PDAC from normal pancreas and pancreatitis tissue samples [22]. Quantitative RT-PCR was used to confirm the findings of the microarray.

Some of the aberrant miRNAs reported by those studies may play an important role in genesis and metastasis of PDAC. Overexpression of miR-221 may be essential for the platelet-derived growth factor (PDGF)-mediated epithelial-mesenchymal transition phenotype, migration, and growth of pancreatic cancer cells [29]. The mRNA expression level

of sel-1-like (SEL1L), a tumor suppressor gene, was found to correlate inversely with the expression of hsa-mir-143, hsa-mir-155, and hsa-mir-223 [31]. Functional analysis revealed that hsa-mir-155 acted as a suppressor of SEL1L in PDAC cell lines. Wu et al. confirmed the upregulation of miR-424-5p expression level in PDAC cells by quantitative RT-PCR and found that the high expression of miR-424-5p suppressed the expression of cytokine-induced signaling 6 (SOCS6), leading to increased proliferation, migration and invasion of pancreatic cancer cells, and inhibited cell apoptosis [37]. Zhang et al. identified cholecystokinin-B receptor (CCKBR) and B cell lymphoma (Bcl-2) as targets of miR-148a, which acted as a tumor suppressor, in the regulation of pancreatic cancer growth and apoptosis [43].

Szafranska et al. investigated the expression of 377 miRNAs in snap-frozen surgical resection pancreatic tissue samples from normal pancreas, chronic pancreatitis (CP), and PDAC. A pancreatic miRNome was established by miRNA arrays and confirmed by quantitative RT-PCR. They found that the expression of some miRNAs, such as miR-29c, miR-96, miR-143, miR-148b, and miR-150, was dysregulated in both CP and PDAC samples, whereas miR-196a, miR-196b, miR-203, miR-210, miR-222, miR-216, miR-217, and miR-375 were aberrantly expressed only in the PDAC samples. The authors concluded that a combination of miR-217 and -196a was able to discriminate normal pancreas, CP, and cancerous tissues [33]. Zhang et al. analyzed miRNA expression of 10 pancreatic cancer cell lines and 17 pairs of pancreatic cancer/normal tissue. The author reported that eight miRNAs, including miR-196a, miR-221, and miR-222, were significantly upregulated in most PDAC tissues and cell lines. The incidence of upregulation of these eight genes between normal control subjects and tumor cells or tissues ranged from 70% to 100% [34].

Ohuchida et al. obtained the miRNA expression profiles of pancreatic cancer cell line CAPAN-1 by microarray analysis. Compared with immortalized human pancreatic ductal epithelial cell line, 8 miRNAs (including miR-10a, miR-17-5p, miR-92, et al.) were upregulated and 2 miRNAs (miR-450 and miR205) were downregulated in CAPAN-1 cells. The microarray data was then confirmed with quantitative RT-PCR analysis. Microdissection analyses revealed that miR-10a was overexpressed in pancreatic cancer cells isolated from a subset of primary tumors (12 of 20, 60%) compared with precursor lesions and normal ducts. And further vitro experiments demonstrated that miR-10a may be involved in the invasive potential of PDAC cells partially via suppression of HOXA1 [38].

In a recent study, Zhang et al. reported a novel mechanism through which increased zinc mediated by the zinc importer ZIP4 could transcriptionally upregulate the expression level of miR-373 in PDAC cells to promote tumour growth. Higher expression of miR-373 was regulated by the zinc-dependent transcription factor CREB, and it enhanced cell proliferation, invasion, and tumour growth through negative regulations against TP53INP1, LATS2, and CD44 [39].

Pancreatic cancer is characterized by a dense stromal reaction. There is accumulating evidence that pancreatic stellate cells (PSCs) promote the progression of pancreatic cancer. In a study focusing on the relationship between PSCs and PDAC, the expression level of miR-210 in PDAC cells was significantly induced by coculturing with PSCs [41]. This upregulation may be attenuated by inhibition of ERK and PI3K/Akt pathways, and the inhibition of miR-210 expression decreased migration, decreased the expression of vimentin and snail-1, and increased the membrane-associated expression of β-catenin in PANC-1 cells through coculturing with PSCs.

Panarelli's studies evaluated miRNA expression in pancreatic resection specimens and fine- needle aspiration biopsies. PDAC showed a higher expression of miR-21, miR-221, miR-155, miR-100, and miR-181b than benign lesions (intraductal papillary mucinous neoplasms and nonneoplastic tissues) by qRT-PCR. Microarray analysis of a subset of carcinomas and intraductal papillary mucinous neoplasms confirmed overexpression of miR-21, miR-221, and miR-181b. Cell blocks containing carcinoma showed higher expression of miR-21, miR-221, and miR-196a than those from benign lesions. These results indicated that a select panel of miRNAs may aid in the distinction among pancreatic lesions in cytology specimens [23].

The majority of PDAC overexpress mesothelin (MSLN), which contributes to enhanced proliferation, invasion, and migration. Marin-Muller et al. compared the expression of 95 cancer-associated miRNAs of PDAC cells with overexpressed or low endogenous MSLN levels. RT-PCR result showed a global dysregulation of miRNA expression, with several miRNAs either upregulated (i.e., miR-10b and miR-196a) or downregulated (i.e., miR-198, miR-200c, and miR-155). miR-198 was the most downregulated in PDAC by overexpression of mesothelin, through NF-κB-mediated OCT-2 induction [45]. The authors suggested that miR-198 acts as a central tumor suppressor and modulates the molecular makeup of a critical interactome in PDAC. Reconstitution of miR-198 in pancreatic cancer cells results in reduced tumor growth, metastasis, and increased survival through directly targeting MSLN, PBX-1, and VCP.

Recent explosion in the knowledge of the molecular genesis regarding pancreatic cancer has defined PDAC as a disease with alterations of a wide range of signaling cascades, which is in contrast with certain tumors that are driven by a single oncogene. However, certain signaling pathways and key nodal points act as core genetic alterations and are commonly detected in PDAC cells. Since miRNA alteration is quite informative in pancreatic cancer diagnosis, exploring those commonly deregulated miRNAs and their targeting proteins will help identify those potential targets for future therapy. The miRNAs most frequently reported in the literature exhibiting aberrant expression in PDAC were miR-15b, miR-21, miR-146a, miR-155, miR-181b, miR-196a, miR-200, and miR-221/222 [21, 22, 28, 33, 34, 46] (Table 1).

3. Biomarkers for Early Detection of PDAC

It is generally recognized that PDAC is an insidious disease with no specific early clinical symptoms, except when the primary tumor is located in the head of the pancreas

(obstructive jaundice). A longer interval between the onset of symptoms and the initial diagnosis of PDAC is associated with the disease being first identified at a more advanced stage with poor prognosis. At the time of diagnosis, less than 15% of patients come with surgically resectable disease. The median survival of unresectable PDAC is only 4–6 months. Although the overall 5-year survival of large resected PDAC (median size 3 cm) is only 10%–20%, it is 30%–60% after resection of small PDAC (tumor size ≤ 2 cm) and exceeds 75% when minute PDAC (≤10 mm in size) is resected [52].

Early detection of PDAC would logically require the detection of small lesions. Current noninvasive imaging techniques such as ultrasound, contrast-enhanced multidetector computed tomography, and magnetic resonance imaging are inadequate for the detection of PDAC at an early stage, because they could not reliably detect tumors <1-2 cm in size [53]. Even invasive techniques, such as endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic-ultrasound (EUS) guided fine-needle aspiration (FNA), which are used to distinguish foci of malignant change in the background of CP, present difficulties.

Serum and plasma remain the most easily accessible samples for diagnostic testing and, hence, are an attractive medium for biomarker testing to screen early-stage diseases. To date, the clinical role of PDAC markers for diagnosis is still limited; meanwhile, the development of minimally invasive biomarker assays for early detection is urgently needed. Several reports indicated that miRNA expression profiles may be useful in diagnosis of specific cancer types [15, 54–63]. For example, Lawrie et al. found that circulating miR-21 was significantly overexpressed in sera from diffuse large B cell lymphoma patients. High expression levels of miR-21 were found to be associated with improved relapse-free survival times.

Kong et al. found that three serum miRNAs, including miR-196a, were differentially expressed in PDAC compared with control groups. Serum miR-196a could be a potential noninvasive marker for PDAC prognosis and selection for laparotomy [35]. Another investigation by Wang et al. showed that the expression levels of four miRNAs in plasma—miR-21, miR-210, miR-155, and miR-196a—were significantly higher in patients with PDAC than in a healthy control group [24].

Li et al. measured 735 circulating miRNAs in PDAC case and control sera [64]. miR-1290 was found to show the best diagnostic performance among all the significantly elevated circulating miRNAs, yielding an area under curve (AUC) of 0.96 [95% confidence interval (CI), 0.91–1.00], 0.81 (0.71–0.91), and 0.80 (0.67–0.93), for subjects with pancreatic cancer relative to healthy controls, subjects with chronic pancreatitis, and pancreatic neuroendocrine tumors, respectively. Kawaguchi et al. found that plasma miR-221 concentrations were significantly higher in PDAC patients than those in benign pancreatic tumors and controls [30]. Furthermore, PDAC patients with high plasma miR-221 concentrations showed significant correlation with distant metastasis and nonresectable status.

Early diagnosis for PDAC requires markers with high sensitivity and specificity. The standard serum marker, salivated Lewis blood group antigen CA19-9, is widely used, but its use is limited to monitoring responses to therapy, not as a diagnostic marker [65, 66]. Recent research results from our group indicate that sera or plasma from patients with PDAC has a unique miRNA expression pattern compared with normal control as well as CP [67, 68]. The combination of miR-16, miR-196a, and CA19-9 was more effective for discriminating PDAC from non-PDAC (normal and CP) with a sensitivity of 92.0% and a specificity of 95.6%. These studies suggest that the amount of miRNAs in serum may have the potential as diagnostic biomarkers for PDAC [35]. miR-210 also has been detected in sera of PDAC patients, while it was expressed at levels that were fourfold higher than in normal controls [42].

Wang et al. [69] investigated miRNA expression in peripheral blood mononuclear cells (PBMCs) in healthy, benign pancreatic/peripancreatic disease (BPD) and PDAC cohort, respectively. Using the method of sequencing technology and quantitative RT-PCR, they found that miR-27a-3p level in PBMCs could discriminate PDAC from BPD. Frampton et al. further examined the miRNA profiles in PBMCs from PDAC patients, based on the theory that circulating blood cells monitors the patients' physiological state and response by altering their transcriptome [70]. They confirmed that miR-27a-3p was upregulated in PBMCs isolated from PDAC patient blood samples and the combination of PBMC miR-27a-3p with serum CA19-9 levels improved diagnostic accuracy.

Sometimes it can be difficult to distinguish malignant and benign lesion on the pancreas with conventional imaging techniques such as CT (computed tomography), MRI (magnetic resonance imaging), and abdominal B-ultrasound. The accuracy of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) biopsy is always affected by the location and size of the lesion, the quantity of tissue obtained, the quality of the histology, et al. A molecular analysis of miRNA expression may improve the diagnosis accuracy. Szafranska et al. used qRT-PCR to quantify miRNA levels in FNA samples and compared the results with a training set consisting of frozen macrodissected pancreatic samples. The authors reported that a combination of miR-196a and miR-217 biomarkers had the ability to distinguish between healthy tissue, PDAC, and CP in the training set as well as segregate PDAC FNA samples from other FNA samples [36]. Hanoun et al. measured the level of DNA methylation of EUS-FNA samples from PDAC and CP patients. Hypermethylation of the DNA region encoding miR-148a led to the inhibition of its gene expression in preneoplastic pancreatic intraepithelial neoplasia (PanIN) [44]. The authors suggested that this phenomenon of hypermethylation can differentiate PDAC from CP and the hypermethylated DNA region encoding miR-148a can serve as an ancillary marker for the differential diagnosis of PDAC and CP.

In some recent studies, miRNAs are also found to be useful as diagnostic markers for some precursor lesions of PDAC. Caponi et al. quantified the expression of three candidate miRNAs (miR-21, miR-155, and miR-101) by quantitative RT-PCR in 86 laser-microdissected intraductal papillary mucinous neoplasms (IPMNs) specimens [71]. They found that miR-21 and miR-155 were upregulated in invasive

IPMNs compared with noninvasive IPMNs and in noninvasive IPMNs compared with normal tissues. However, miR-101 levels were significantly higher in noninvasive IPMNs and normal tissues compared with invasive IPMNs. Further multivariate analysis showed that high-miR-21 expression emerged as an independent prognostic biomarker in invasive IPMNs with bad survival. Lubezky et al. [72] also found miRNAs useful to identify IPMN with high risk for malignant transformation. They analyzed the expression patterns of 846 human miRNAs with microRNA microarray in 55 tissues that range from low-grade dysplastic IPMN to PDAC. Expression of 15 miRNAs, including miR-217, miR-216a, miR-21, and miR-155, was significantly different between two IPMN subgroups: low- and moderate-grade dysplastic IPMNs versus high-grade dysplastic IPMN and invasive cancer with IPMN. Pancreatic cysts are a group of lesions with heterogeneous malignant potential. Farrell et al. [73] compared the expression of miRNAs in benign, premalignant, and malignant cysts fluidusing a whole-genome expression array analysis. The results showed that pancreatic cyst fluids miR-21 and miR-221 are associated with invasive cancer. miR-221 was expressed at significantly higher levels in malignant cysts compared with benign or premalignant cysts and miR-21 was also expressed at significantly higher levels in premalignant and malignant cysts.

Despite the fact that the diagnostic value of miRNAs expression aberration in PDAC has been extensively studied in recent years, differences in measurement platforms and lab protocols can render gene expression levels incomparable. Ma et al. [74] in their recent metareview, which included a total of 538 tumors and 206 noncancerous control samples, identified a statistically significant miRNA metasignature of seven up- (including miR-21, miR-155, and miR-221) and three downregulated miRNAs (miR-217, miR-148a, and miR-375).

In conclusion, no PDAC marker has been shown to be useful in the early detection of an asymptomatic population so far. Serum and plasma miRNAs, for example, miR-21, miR-155, miR-210, and miR-196a, are promising biomarkers for early detection of PDAC, especially with the combination of serum CA19-9 levels.

4. Prognosis and miRNAs

Poor survival is a hallmark feature of PDAC. Several studies have suggested prognostic significance of miRNAs expression profiles in PDAC. For example, miR-21 appears to confer chemoresistance to PDAC cell lines; strong miR-21 expression was predictive of poorer outcomes compared with absent or faint/focal miR-21 expression in patients with node-negative PDAC (median 15.2 versus 27.7 months) [25]. Jamieson et al. [75] also found that miR-21 was associated with poor prognosis. In their study, they performed the global miRNA microarray expression profiling of frozen PDAC tissue from 48 patients confirmed by RT-PCR analysis. After a further validation set of 24 patients, they found that high expression of miR-21 and reduced expression of miR-34a were significantly associated with poor overall survival. Frampton et al. [76] in their recent study found that, in 91 PDAC samples from patients, high level of a combination of miR-21, miR-23a, and miR-27a was associated with shorter survival times after surgical resection.

miR-200c, a member of the miR-200 family, may be a valuable prognostic marker for PDAC. The expression of miR-200c in PDAC shows a wide range. While strong expression of miR-21 predicts limited survival in PDAC patients, high expression of miR-200c is a sign of good prognosis [49]. Specifically, the median survival times and five-year survival rates were 42 months and 33.5% in the high miR-200c expression group and 19 months and 11.2% in the low miR-200c expression group. In a recent study, researchers suggested that miR-200c overexpression downregulates transmembrane mucins MUC4 and MUC16 in pancreatic cancer cells by directly targeting the mRNA coding sequence of each, resulting in reduced levels of MUC4 and MUC16 mRNA and protein, which are associated with tumor progression and metastatic potential in human PDAC [50].

Elevated levels of miR-155, miR-203, miR-210, and miR-222 expression in PDCA were significantly associated with increased risk (6.2-fold) of death compared to patients with reduced expression of these miRNAs [32]. A subgroup of 6 miRNAs (miR-452, miR-105, miR-127, miR-518a-2, miR-187, and miR-30a-3p) was found to be able to distinguish long-term survivors with node-positive disease from those dying within 24 months [22]. miR-196a-2 may also be a negative survival predictor; median survival for pancreatic cancer patients has been shown to be 14.3 versus 26.5 months, depending on miR-196a-2 expression level. Elevated expression of miR-196a-2 was predictive of median survival differing by about a year among pancreatic cancer patients. In addition, increased expression of miR-155, miR-203, miR-210, and miR-222 was also found to be significantly associated with poorer survival of PDAC patients [32, 77]. Zhu et al. reported that reduced miR-218 in PDAC tissues was correlated with tumor progression and might be an independent poor prognostic factor for patients [78]. In a recent study, Zhao et al. found that miR-130b was significantly downregulated in 52 pancreatic cancer tissues (compared with paracancerous tissues) and five cell lines [79]. Furthermore, the deregulated miR-130b was correlated with worse prognosis, increased tumor size, late TNM stage, lymphatic invasion, and distant metastasis. Dual luciferase assay revealed that STAT3 may be one direct target of miR-130b.

5. Chemosensitivity and Radiosensitivity

miRNAs have been shown to induce changes in the chemosensitivity or radiosensitivity of PDAC cells in a variety of settings, with certain miRNAs identified to indicators for chemotherapy efficacy (Figure 1). miR-21, for example, appears to convey chemoresistance to PDAC cells. miR-21 previously has been shown to be significantly upregulated in PDAC, whereas stronger expression of miR-21 is associated with poorer survival of this cancer [25], indicating its oncogenic properties. Forced expression of miR-21 increases proliferation and invasion of PDAC cell lines, and this appears

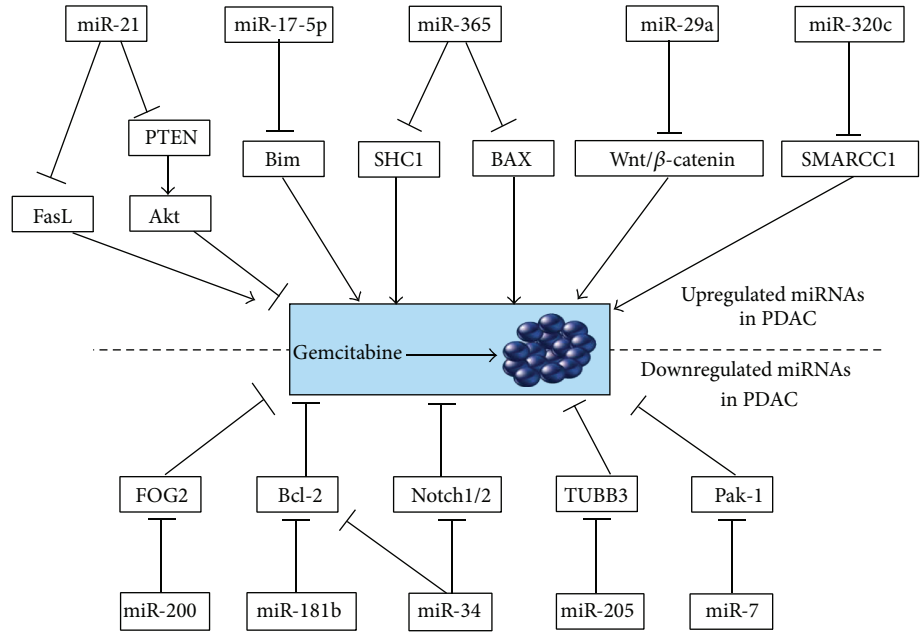

Figure 1: Influences of miRNAs on the gemcitabine treatment in PDAC. miRNAs that are upregulated in PDAC inhibit gemcitabine-sensitive associated genes, such as PTEN, Bim, SHC1, BAX, and SMARCC1. Conversely, miRNAs that are downregulated in tumors inhibit gemcitabine-resistant associated genes, such as Pak-1, TUBB3, Notch1/2, Bcl-2, and FOG2.

to occur through target inhibition of the phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4), presence of tropomyosin 1 (TPM1), and tissue inhibition of metalloproteinases-3 (TIMP3), thereby indirectly inducing expression of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor (VEGF). Meanwhile, miR-21 appears also to induce chemoresistance to gemcitabine in PDAC cell lines. For example, when PDAC cell lines PANC-1, LPc111, and LPc006 were transfected with the miR-21 precursor, those cells were resistant to gemcitabine treatment, showing reduced apoptosis and increased proliferation [26, 80]. In contrast, the inhibition of miR-21 function induced more apoptosis and decreased proliferation in PDAC cell line SUIT-2, which expresses relatively high levels of miR-21 [26]. In another study, Hwang et al. [27] suggested that miR-21 might be a useful biomarker for chemosensitivity, as their research showed that the PDAC cells with lower miR-21 expression had higher chemosensitivity to 5-fluorouracil (5-FU). miR-21 leads to downregulation of PTEN and a more active signaling through the PI3K/Akt/mTOR pathway. Modulation of apoptosis, Akt phosphorylation, and expression of genes involved in invasive behavior may contribute to the role of miR-21 in gemcitabine chemoresistance. Wang et al. confirmed that FasL was a direct target of miR-21. They found that increased FasL expression following gemcitabine treatment could induce cancer cell apoptosis, whereas the ectopic expression of miR-21 partially protected the cancer cells from gemcitabine-induced apoptosis [81].

Hamada et al. found that miR-365 was highly expressed in invasive PDAC and could induce gemcitabine resistance in pancreatic cancer cells. The authors suggested that miR-365 may induce chemoresistance through directly targeting adaptor protein Src homology 2 domain containing 1 (SHC1) and apoptosis-promoting protein BAX [82]. The siRNA-based knockdown of SHC1 and BAX increased gemcitabine resistance, indicating the miR-365/SHC1/BAX axis might influence the survival of pancreatic cancer cells.

Nagano et al. investigated the relationship between miR-29a expression and the response to gemcitabine in PDAC cells [83]. MIAPaCa-2 and PSN-1 cells transfected with anti-miR-29a showed significantly lower resistance to gemcitabine. Putative target molecules showed overexpression in the transfected cells including Dkk1, Kremen2, and sFRP2 and lower activation of the Wnt/beta-catenin signaling pathway. The authors suggested that activation of the Wnt/beta-catenin signaling pathway mediated the miR-29a-induced resistance to gemcitabine in PDAC cell lines

Iwagami et al. reported that high expression of miR-320c in MiaPaCa2 induced resistance to gemcitabine [84]. miR-320c-related resistance to gemcitabine was mediated through SMARCC1, a core subunit of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex. Further clinical examination revealed that only SMARCC1-positive patients benefited from gemcitabine therapy with regard to survival after recurrence.

In a recent study, Yan et al. reported that transfected PDAC cell lines Panc-1 and BxPC3 with miR-17-5p inhibitor showed growth inhibition, spontaneous apoptosis, higher caspase-3 activation, and increased chemosensitivity to gemcitabine [85]. miR-17-5p inhibitor upregulated Bim protein expression in a dose-dependent manner without changing the Bim mRNA level, proving that miR-17-5p negatively regulates Bim at the posttranscriptional level.

miR-200 is a potential tumor suppressor that plays important roles in cancer metastases [86, 87]. Like miR-21, miR-200 may be involved in chemoresistance, or in this case

chemosensitivity. Ali et al. reported that decreased expression of miR-200 and increased expression of miR-21 are associated with gemcitabine resistance in PDAC cells. Interestingly, treatment with curcumin, a major chemical component in turmeric, a spice commonly used in Indian cooking, resulted in upregulation of miR-200 expression and downregulation of miR-21 expression [88].

Several studies identified miRNAs which may sensitize PDAC to chemotherapy or radiotherapy. Although gemcitabine-resistant PDAC cell sublines (SW1990/GR and CFPAC-1/GR) expressed higher levels of miRNA-181b, Cai et al. found that gemcitabine induced higher levels of apoptosis in PDAC cells transfected with miRNA-181b mimics [89]. Nude mouse xenograft assay data showed that miR-181b transfection also sensitized the cells to gemcitabine treatment in vivo. Further study showed a reduced BCL-2 expression following miR-181b transfection but an enhanced caspase-3 activity in miRNA-181b mimic-transfected PDAC cells, indicating that miRNA-181b may sensitize PDAC cells to gemcitabine by targeting BCL-2. The study by Singh et al. identifies a series of miRNAs which were either upregulated (e.g., miR-146) or downregulated (e.g., miR-205, miR-7) in gemcitabine resistant MIA PaCa-2 cancer cells and clinical metastatic pancreatic cancer tissues [90]. Transfection with miR-205 resulted in the restoration of chemosensitivity to gemcitabine with decreased expression of stem cell markers OCT3/4 and CD44 and chemoresistance marker class III b-tubulin. miR-34 also appears to sensitize PDAC cells to chemotherapy and radiotherapy. It has been reported that expression of miR-34, which shows tumor suppressive qualities, is significantly lower in PDAC cell lines than in normal pancreatic ductal epithelial cell lines [91]. miR-34 expression normally is regulated by the tumor suppressor gene p53 [92], but it also can be inactivated by aberrant CpG methylation in cancer [93]. Evidences showed that restoration of miR-34 expression induces a G1 cell cycle arrest and apoptosis in some malignancies, including PDAC. In another study, Wang et al. found that miR-23b overexpression inhibited radiation-induced autophagy and sensitized PDAC cells to radiation. They suggested that, in PDAC, reduced miR-23b level increases levels of its target AGT12 and autophagy to promote radioresistance [94].

6. miRNAs as Potential Therapeutic Targets in PDAC

As discussed above, many miRNAs downregulate genes that are highly relevant to PDAC and contribute to disease progression; thus, chemically modified antisense oligonucleotides or ectopic expression of miRNAs might be considered for therapy. Since one single miRNA might potentially affect several target genes, artificially increasing or decreasing the expression signature of a given miRNA offers interesting therapeutic possibilities.

RNA interference (RNAi) was identified in *C. elegans* in 1998 [95] and in mammalian cells in 2001 [96]. Since then, RNAi has generated increasing interest and publications in diverse research areas. The main problem involved in RNAi-based gene therapy is the delivery of the effector molecule, which should preferably be controllable, sustained, and tissue-specific. Several groups have opted for nonviral delivery of synthetic miRNA molecules. miRNA mimics or miRNA antagomirs can be repeatedly delivered locally or systemically, causing transient suppression of target gene expression [97]. Morrissey et al. intravenously injected mice carrying replicating HBV with a stabilized siRNA targeting the HBV RNA that had been incorporated into a specialized liposome to form a stable nucleic-acid-lipid particle (SNALP). The improved efficacy of siRNA-SNALP was compared with unformulated siRNA leading to a longer half-life in plasma and liver. RNAi incorporated into SNALPs could protect them from degradation, prevent immunostimulation, and facilitate their uptake in endosomes [98]. In addition, $2'$O-methyl modifications increase the stability of synthetic molecules, preventing off-targeting [99].

Aberrant miRNA expression in PDAC oncogenically affects cancer suppressor genes, causing subsequent effects on PDAC cell proliferation, apoptosis, and metastasis. For example, Tsuda et al. found that miRNA (Gli-1-miRNA-3548) and its corresponding duplex (Duplex-3548) significantly inhibited proliferation of Gli-1$^+$ ovarian (SK-OV-3) and pancreatic (MiaPaCa-2) tumor cells. The miRNAs mediated delayed cell division and activation of late apoptosis in MiaPaCa-2 cells [100, 101]. miR-96 directly targets the KRAS oncogene, and ectopic expression of miR-96 can reduce pancreatic cell proliferation, migration, and invasion, suggesting its therapeutic potential in PDAC [48].

Other miRNAs with oncogenic or tumor suppressor functions, including let-7, miR-21, miR-27a, miR-31, miR-200, and miR-221, could be used as novel therapeutic agents for PDAC. Several studies reported that antisense to miR-21 and miR-221 could improve the chemosensitivity of gemcitabine, and the antisense-gemcitabine combinations resulted in significant cell killing under various conditions [26, 102]. Overexpression of miR-204, either by a miR-204 mimic or by triptolide treatment, downregulates myeloid cell leukemia-1 (Mcl-1) by directly binding to the Mcl-1 $3'$ UTR and causes a subsequent decrease in cell viability and pancreatic cancer cell death [103]. Yan et al. reported that miR-20a could regulate Stat3 at the posttranscriptional level, resulting in inhibition of cell proliferation and invasion of pancreatic carcinoma [47].

Both the inhibition of miR-31 in AsPC-1 and HPAF-II PDAC cells with high endogenous expression and forced expression of miR-31 in MIA PaCa-2 with low endogenous levels led to reduced cell proliferation, migration, and invasion. More importantly, in AsPC-1 cells, further enhancement of miR-31 also resulted in reduced cell migration and invasion, implicating that the level of miR-31 is critical for these phenotypes [104]. miR-27a may play an oncogenic role by targeting Spry2 and modulating the malignant behaviors of PDAC cells. Spry2 protein, which has a low expression level in pancreatic adenocarcinoma, was upregulated by transfection with a miR-27a inhibitor [40]. Torrisani et al. found that let-7 expression is strongly reduced in PDAC samples (compared with adjacent tissues). Restoring let-7 levels in cancer-derived cell lines, by transfection with

plasmid-based synthetic miRNAs or by lentiviral transduction, strongly inhibited cell proliferation, K-ras expression, and mitogen-activated protein kinase activation. However, intratumoral gene transfer or implantation of Capan-1 cells stably overexpressing let-7 failed to impede tumor growth progression [51].

Frampton et al. analyzed the combined effects of altered activities of miRNAs in PDAC cell lines and in PDAC samples from patients. They found that 3 miRNAs (miR-21, miR-23a, and miR-27a) may act as cooperative repressors of a network of tumor suppressor genes that included PDCD4, BTG2, and NEDD4L. Inhibition of miR-21, miR-23A, and miR-27A had synergistic effects in reducing proliferation of PDAC cells in culture and growth of xenograft tumors in mice. The level of inhibition was greater than that of inhibition of miR-21 alone [76].

These studies opened a new perspective and provided early steps for miRNA replacement therapy for PDAC. However, before miRNA based therapeutics enter clinics, hurdle issues such as specific delivery to certain cells of interest, safety, and pharmacokinetics are warranted to be addressed.

7. Conclusion

It is well established that miRNAs are vital factors in a wide variety of biological processes, including development, cellular proliferation, invasion, and apoptosis. In PDAC, miRNAs showed aberrant processing and expression signatures. Identification of unique patterns of dysregulated miRNA expression in PDAC provides valuable information that may serve as molecular biomarkers for tumor diagnosis, disease prognosis, and prediction of therapeutic responses. Although some miRNAs have been found to be associated with proliferation, invasion, and prognosis of PDAC, the precise mechanisms controlling the processes mentioned earlier remain elusive. Further investigation should explore the interpretation of miRNA profiling data and regulatory functions of miRNA in pancreatic cancer development and progression.

So far, the researches on miRNA's potential clinical usage are mainly conducted at molecular level and retrospectively with relatively small sample sizes. Although the results are promising, large scale prospective validation studies to test its diagnostic and prognosis value are needed. Therefore, miRNAs can serve as a valuable diagnostic marker and therapeutic target for pancreatic cancer in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Tao Sun and Xiangyu Kong contributed equally to this work.

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Research Article

ATP-Binding Cassette Genes Genotype and Expression: A Potential Association with Pancreatic Cancer Development and Chemoresistance?

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Genetic polymorphisms in ABC (ATP-binding cassette) transporter genes are associated with differential responses to chemotherapy in various cancers including pancreatic cancer. In this study, four SNPs in the ABCB1, ABCC1, and ABCG2 genes were investigated in normal and pancreatic cancerous specimens. The expression of the three transporters was also analyzed. The TT genotypes of G2677T and C3435T in ABCB1 gene were associated with lower risk of developing pancreatic cancer ($P = 0.013$, OR = 0.35 and $P = 0.015$, OR = 0.29, resp.). To our knowledge, this is the first report of the common polymorphisms in the ABCB1 gene affecting the genetic risk of developing pancreatic cancer. Moreover, the expression of ABCB1 in 2677TT and 3435TT carriers was lower compared to the wild-type homozygotes and heterozygotes. A cell viability assay, using standard pancreatic cancer cell lines, revealed that the ABCB1 2677TT-3455TT haplotype was more sensitive than the other haplotypes to gemcitabine. *Conclusion*. Polymorphisms in ABCB1 G2677T and G3435T were associated with differential susceptibility to pancreatic cancer and may predict responses to chemotherapy.

1. Introduction

Pancreatic cancer is the 10th most commonly diagnosed cancer and the 4th leading cause of cancer death in the US [1, 2]. Due to the lack of symptoms and early detection measures, pancreatic cancer is typically diagnosed at a late stage; only 10% to 15% of patients are diagnosed at a relative early stage, when surgical removal of tumor remains possible. However, because of the aggressive nature of pancreatic cancer, the recurrence rate remains very high. For up to 80% of postoperative pancreatic cancer patients, cancer reoccurs within two years after surgery. Chemotherapy is the main treatment for locally advanced, metastatic, and recurrent pancreatic cancer, but the efficacy is limited [3]. Even with gemcitabine, the golden standard for advanced pancreatic cancer treatment, the objective tumor response rate is only about 15–20% and the median survival in randomized trials is only 5–6.7 months [3]. In fact, pancreatic cancer has the highest mortality rate of all the major cancers—only 5% of patients will survive for more than five years, and the survival rate has not improved in nearly 40 years [1]. Moreover, pancreatic cancer incidence is expected to increase due to demographic changes and a number of lifestyle factors, specifically smoking, added sweeteners, and eating diets heavy in animal products [4, 5]. Understanding pancreatic cancer susceptibility and mechanism(s) of limited efficacy of chemotherapy is critical to the fight against this deadly disease.

Table 1: (a) Sample characteristics (genotyping). (b) Sample characteristics (expression).

(a)

	Age (yr)	Sex *n* (%)	Race *n* (%)
NOR (76)	56.8 ± 1.9	F 41 (53.9) M 35 (46.1)	Euro-Am 58 (76.3) Afri-Am 12 (15.8)
MAL (76)	65.1 ± 1.5	F 39 (52.0) M 36 (48.0)	Euro-Am 63 (82.9) Afri-Am 8 (10.5)

(b)

	Age (yr)	Sex *n* (%)	Race *n* (%)
NOR (60)	57.0 ± 2.1	F 33 (55.0) M 27 (45.0)	Euro-Am 46 (76.7) Afri-Am 10 (16.7)
MAL (60)	64.1 ± 2.0	F 32 (53.3) M 27 (45.0)	Euro-Am 51 (85.0) Afri-Am 6 (10.0)

One major reason for the limited efficacy of chemotherapy for pancreatic cancer is chemoresistance. Overexpression of ATP-binding cassette (ABC) transporters has been documented to play an important role in the development of chemoresistance in various cancers [6–8]. ABC transporters represent a superfamily of membrane proteins that actively transport a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, and drugs. Overexpression of ABC transporters leads to increased drug efflux thereby reducing intracellular drug levels and causing drug resistance. Of the 49 human ABC transporters, 15 are implicated in conferring resistance to chemotherapeutic agents in various cancers, and the most intensively characterized members are multidrug resistance 1 (MDR1 or P-glycoprotein, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2) [7]. Elevated expression of ABCB1, ABCG2, and ABCCs mRNA in pancreatic adenocarcinomas compared to normal pancreas has been reported [9–12], but correlation with clinical aggressiveness of the tumor remains controversial [11, 12].

Polymorphisms in ABC transporters have been intensively investigated and linked with varied expression of efflux pumps in different tissue compartments, altered drug levels, and host susceptibilities to several diseases. For pancreatic cancer, a recent study in pancreatic cancer survivors found that a single nucleotide polymorphism (SNP) in ABCG2 (rs2231164) correlated with pancreatic cancer survival; patients carrying the AG/GG genotypes exhibited better survival than those carrying the AA genotype [13]. Another study in patients who were treated with gemcitabine before surgery showed that two SNPs in ABCC2 and ABCC5 were associated with overall survival, and the ABCC2 G40A GG genotype was associated with poor histological response to gemcitabine [14]. The SNP ABCB1-G2677T was also reported to correlate with drug response in patients receiving adjuvant chemotherapy with gemcitabine [15]. However, very few studies have investigated the role of ABC transporter gene polymorphisms in pancreatic cancer development and whether ABC transporter genotypes are correlated with the expression of transporters in pancreas. In this study, the role of ABC transporters in pancreatic cancer development and chemoresistance was investigated. Specifically, the genetic polymorphisms of ABCB1, ABCC1, and ABCG2 in both normal and pancreatic cancerous specimens were analyzed. Expression levels of the efflux pumps were examined and correlated to the SNPs. The potential correlation of ABC transporter genotype with chemotherapy sensitivity was also investigated in several pancreatic cancer cell lines.

2. Materials and Methods

2.1. Patient Information and Sample Collection. Frozen pancreatic resection specimens were purchased from the US Cooperative Tissue Network (CHTN) (Birmingham, AL, USA) with nonidentifiable codes. The pathology and clinical information of the purchased samples were retrospectively collected from specimen information sheets. For genotyping analysis, a total of 152 samples (one pancreatic resection sample per patient) were included in the study and 120 were used for gene expression analysis. Half of the samples were from normal pancreatic tissue (NOR), and the other half were from malignant pancreatic cancerous specimens (MAL). More than 75% of the samples were from European Americans, and others were from either African Americans or ethnic unknowns (Tables 1(a) and 1(b)). For the malignant samples, the majority of them (90%) are diagnosed as pancreatic adenocarcinoma.

2.2. DNA Extraction and Genotyping. Four SNPs, rs2032582 (ABCB1 G2677T), rs1045642 (ABCB1 C3435T), rs4148330 (ABCC1 G-260A), and rs2231142 (ABCG2 C421T), were selected according to the following criteria: a minor allele frequency of the SNP greater than 0.1 in dbSNP and the SNP had been reported to affect expression/function of the ABC transporter or had been associated with cancer risk/clinical outcome in prior studies. Genomic DNA was extracted from pancreatic tissues or cells using QIAamp DNA kits (Qiagen, Valencia, CA, USA). Polymorphisms were detected with TaqMan SNP genotyping assays on a 7900HT Fast Real-Time PCR machine (Applied Biosystems/Life Technologies, Grand Island, NY, USA). The allelic discrimination analysis was verified with the real-time PCR results to ensure the genotyping accuracy.

2.3. RNA Isolation and Real-Time qRT-PCR. Total RNA was isolated from frozen pancreatic tissues or cells using RNeasy kits (Qiagen) and reverse transcribed into cDNA using RT^2 First Strand kits (SABiosciences/Qiagen). SYBR green-based

Table 2: Genotypic frequency in the whole study population.

Genotypes	NOR (n = 76) N (freq.%)	MAL (n = 76) N (freq.%)	OR (95% CI)	P value
ABCB1 G2677T/A			0.29 (0.11~0.79)	0.015
GG	27 (35.5)	28 (36.8)	TT versus (GT+GG)	
GT	31 (40.8)	38 (50.0)		
TT	18 (23.7)	6 (7.9)		
TA	0 (0)	2 (2.6)		
GA	0 (0)	2 (2.6)		
ABCB1 C3435T			0.35 (0.15~0.80)	0.013
CC	22 (28.9)	21 (27.6)	TT versus (CT+CC)	
CT	31 (40.8)	45 (59.2)		
TT	23 (30.3)	10 (13.2)		
ABCC1 G-260A			1.49 (0.62~3.60)	0.376
GG	10 (13.2)	14 (18.4)	GG versus (GA+AA)	
GA	35 (46.0)	32 (42.1)		
AA	31 (40.8)	30 (39.5)		
ABCG2 C421T			5.13 (0.24~108.70)	0.294
CC	61 (80.3)	62 (81.6)	TT versus (CT+CC)	
CT	15 (19.7)	12 (15.8)		
TT	0 (0)	2 (2.6)		

real-time qRT-PCR was performed on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, NC, USA) with gene-specific primers purchased from SABiosciences. The PCR products were verified by gel electrophoresis and melting curve analysis. The housekeeping gene phosphomannomutase 1 (PMM1) was used as the endogenous standard for normalization because, as others had reported [16, 17], we found that the expression of GAPDH, but not PMM1, was increased in pancreatic cancerous specimens.

2.4. Cell Culture and Cell Viability Assay. The human pancreatic cancer cells, BXPC-3, AsPC-1, CFPAC-1, PANC-1, PL-45, MiaPaca-2, and SU86.86, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured as previously described [18]. Briefly, 24 hours after seeding (in 96-well plates), the pancreatic cancer cells were treated with different concentrations of gemcitabine (Eli Lilly Co., Indianapolis, IN, USA) for 48 hours and the cell viability was determined with the CellTiter 96 AQueous One Solution Cell Proliferation assay (Promega, Madison, WI, USA) by following the manufacturer's instructions. The concentration of gemcitabine required to cause 50% growth inhibition (IC_{50}) was calculated using Graphpad Prism 6 software (San Diego, CA, USA).

2.5. Statistical Analysis. The genotyping data were analyzed with PLINK (http://pngu.mgh.harvard.edu/purcell/plink/). The independence of genotype frequencies of the studied SNPs was tested for Hardy-Weinberg equilibrium. Differences in the frequencies of the ABCB1, ABCC1, and ABCG2 gene polymorphisms between NOR and MAL specimens were analyzed using Fisher's Exact tests. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the allelic and genotypic comparisons, following codominant, dominant, and recessive genetic model tests. Unless stated otherwise, the gene expression data were compared using an unpaired t-test. In all instances, results were considered statistically significant at the level of $P < 0.05$.

3. Results

In the total number of 152 samples (121 European Americans) analyzed in this study, the distribution of genotypic and allelic frequencies of all four SNPs met the Hardy-Weinberg equilibrium in both the whole study population and the European American subgroup. Although the allele frequencies of the four SNPs did not differ between the NOR and MAL groups in the whole study population, the mutant allele T in rs2032582 (ABCB1 2677T) tended to have a higher frequency in NOR than MAL European Americans (P = 0.057, data not shown). For the genotypic frequencies, the mutant homozygous genotypes of rs2032582 and rs1045642 (2677TT and 3435TT) in the ABCB1 gene were significantly associated with reduced risks of developing pancreatic cancer in the whole study population and the European American subgroup (Tables 2 and 3, P = 0.015, OR = 0.29 and P = 0.013, OR = 0.35, resp., for the whole study population; P = 0.043, OR = 0.34 and P = 0.033, OR = 0.39, resp., for the European Americans). The distributions of the two SNPs were also statistically significant for the recessive genetic model testing both in the whole study population and in the European American subgroup (P = 0.014 and 0.049 for G2677T; P = 0.017 and 0.035, for C3435T, resp.). No statistical differences were found for the allele and genotype

Table 3: Genotypic frequency in European Americans.

Genotypes	NOR (n = 58) N (freq.%)	MAL (n = 63) N (freq.%)	OR (95% CI)	P value
ABCB1 G2677T/A			0.34 (0.12~0.97)	0.043
GG	16 (27.6)	23 (36.5)	TT versus (GT+GG)	
GT	28 (48.3)	32 (50.8)		
TT	14 (24.1)	6 (9.5)		
TA	0 (0)	1 (1.6)		
GA	0 (0)	1 (1.6)		
ABCB1 C3435T			0.39 (0.16~0.92)	0.033
CC	13 (22.4)	16 (25.4)	TT versus (CT+CC)	
CT	26 (44.8)	37 (58.7)		
TT	19 (32.8)	10 (15.9)		
ABCC1 G-260A			1.96 (0.56–6.91)	0.293
GG	4 (6.9)	8 (12.7)	GG versus (GA+AA)	
GA	29 (50.0)	27 (42.9)		
AA	25 (43.1)	28 (44.4)		
ABCG2 C421T			2.81 (0.11–70.31)	0.530
CC	45 (77.6)	52 (82.5)	TT versus (CT+CC)	
CT	13 (22.4)	10 (15.9)		
TT	0 (0)	1 (1.6)		

frequencies of the other two SNPs between NOR and MAL pancreatic samples.

Expression of ABCB1, ABCC1, and ABCG2 genes was analyzed and all three ABC transporters were found to be significantly increased in MAL specimens compared to NOR pancreases in almost all the study populations/subgroups, except for the expression of ABCC1 in African Americans (Figure 1). Expression of the three ABC transporters was also compared based on their genotypes and no correlations of mRNA expression with genotypes in ABCC1 G-260A and ABCG2 C421T were found (data not shown). However, compared to other genotypes, the mutant homozygotes of ABCB1 2677TT and 3435TT were correlated with significantly reduced expression of ABCB1 in NOR pancreases in both the whole study population and European American subgroup (Figures 2(a) and 2(b)). Similar trends were also seen in the MAL specimens, but the differences were not statistically significant.

Because the ABCB1 G2677T and C3435T were in linkage disequilibrium, the haplotype frequencies of the two SNPs were also compared between NOR and MAL specimens and correlated with mRNA expression in European Americans. The ABCB1 2677TT-3435TT haplotype was significantly associated with reduced risk of developing pancreatic cancer (OR 0.27, 95% CI 0.08 to 0.92, $P = 0.037$) in European Americans, and the expression of ABCB1 was also lower compared to the other haplotypes (Figure 3).

Pancreatic cancer cell lines are commonly used to study the mechanisms of chemoresistance. To investigate whether the protective ABCB1 genotype/haplotype identified in this study was correlated with any functional significance in response to chemotherapy drugs, we analyzed the expression of the ABCB1 gene, the genetic polymorphism of ABCB1, and the sensitivity to gemcitabine in seven pancreatic cancer cell lines. Interestingly, three of the cell lines, MiaPaca-2, BXPC-3, and CFPAC-1, were found to be ABCB1 2677TT and 3435TT homozygotes. The cell viability assay showed that these three cell lines were more sensitive to gemcitabine than PANC-1, SU86.86, PL-45, and AsPC-1, which were either ABCB1 G2677T-C3435T wild-type homozygotes or heterozygotes (Table 4). However, there was no association between ABCB1 mRNA expression and sensitivities to gemcitabine in these cell lines (Table 4).

4. Discussion

Many factors have been linked to the increased risks of developing pancreatic cancer, including age, race, obesity, cigarette smoking, diabetes, chronic pancreatitis, and genetic factors. Indeed, genetic factors play an important role in both familial and sporadic occurrences of pancreatic cancer. Mutations in the high penetrance genes such as BRCA2, PALB2, p16/CDKN2A, PRSS1, SPINK1, and STK11 correlate with a very high lifetime risk of developing pancreatic cancer and may cause as many as 10% of pancreatic cancers in the US [19]. For the nonfamilial (sporadic) pancreatic cancer, a few low penetrance genes have been identified by genome-wide association studies (GWAS). Pancreatic cancer candidate genes have also been reported from studies that examined the biological pathways known to be important in the development of pancreatic cancer, for example, tobacco metabolism, DNA repair, inflammation, and folate metabolism [19, 20].

Genetic variants in ABC transporter genes have been intensively investigated and linked to differential disease

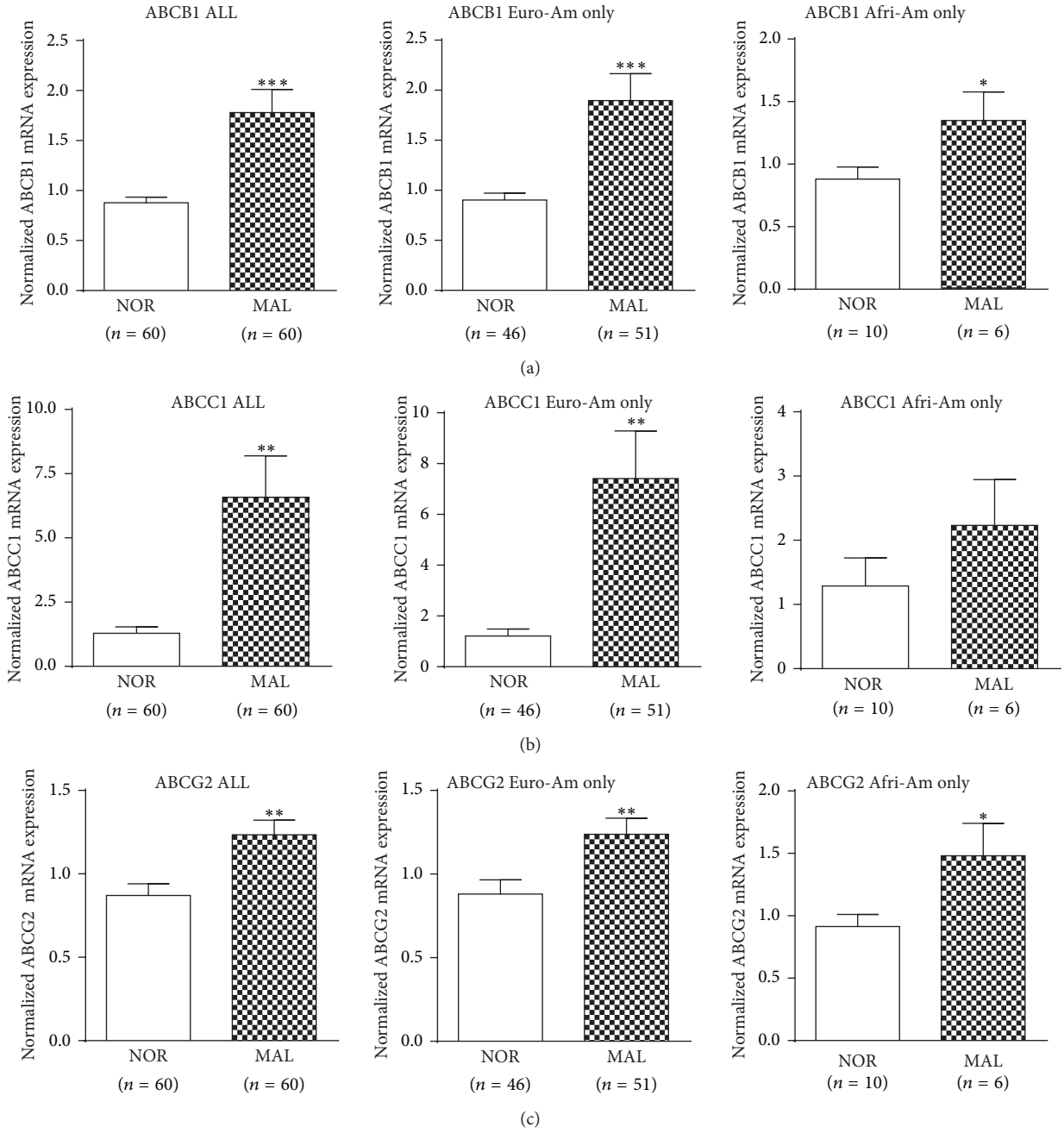

Figure 1: Comparison of ABCB1, ABCC1, and ABCG2 gene expression in normal and pancreatic cancerous tissue. The individual expression levels of the indicated ABC transporters (a–c) were analyzed by real-time qRT-PCR, normalized to mRNA expression of PMM1, and reported as x-fold relative to the expression of a calibrator (a premixed pancreatic RNA sample, set as 1). Values are expressed as means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Euro-Am, European Americans; Afri-Am, African Americans.

susceptibilities and varied responses to therapeutic drugs [7]. In this study, the ABCB1 2677TT and 3435TT genotypes were found to be associated with reduced risk of developing pancreatic cancer in the whole study population (Table 2) and European Americans (Table 3). In fact, combination of the data of European Americans with the ethnic unknowns (considering more than 80% of pancreatic cancer patients in the US are European Americans) showed that the distribution of the ABCB1 G2677T was statistically significant for four of the PLINK tests, including Fisher's Exact test for allelic and genotypic frequencies ($P = 0.043$ and 0.039, resp.), Cochran-Armitage trend test ($P = 0.029$), and recessive genetic model test ($P = 0.020$); for ABCB1 C3435T, Fisher's Exact test for genotypic frequency and recessive genetic model test were statistically significant ($P = 0.028$ and $P = 0.013$, resp.); the P values of Cochran-Armitage trend test ($P = 0.058$) and Fisher's Exact test for allelic frequency ($P = 0.066$) were close to statistical significance. Due to the smaller

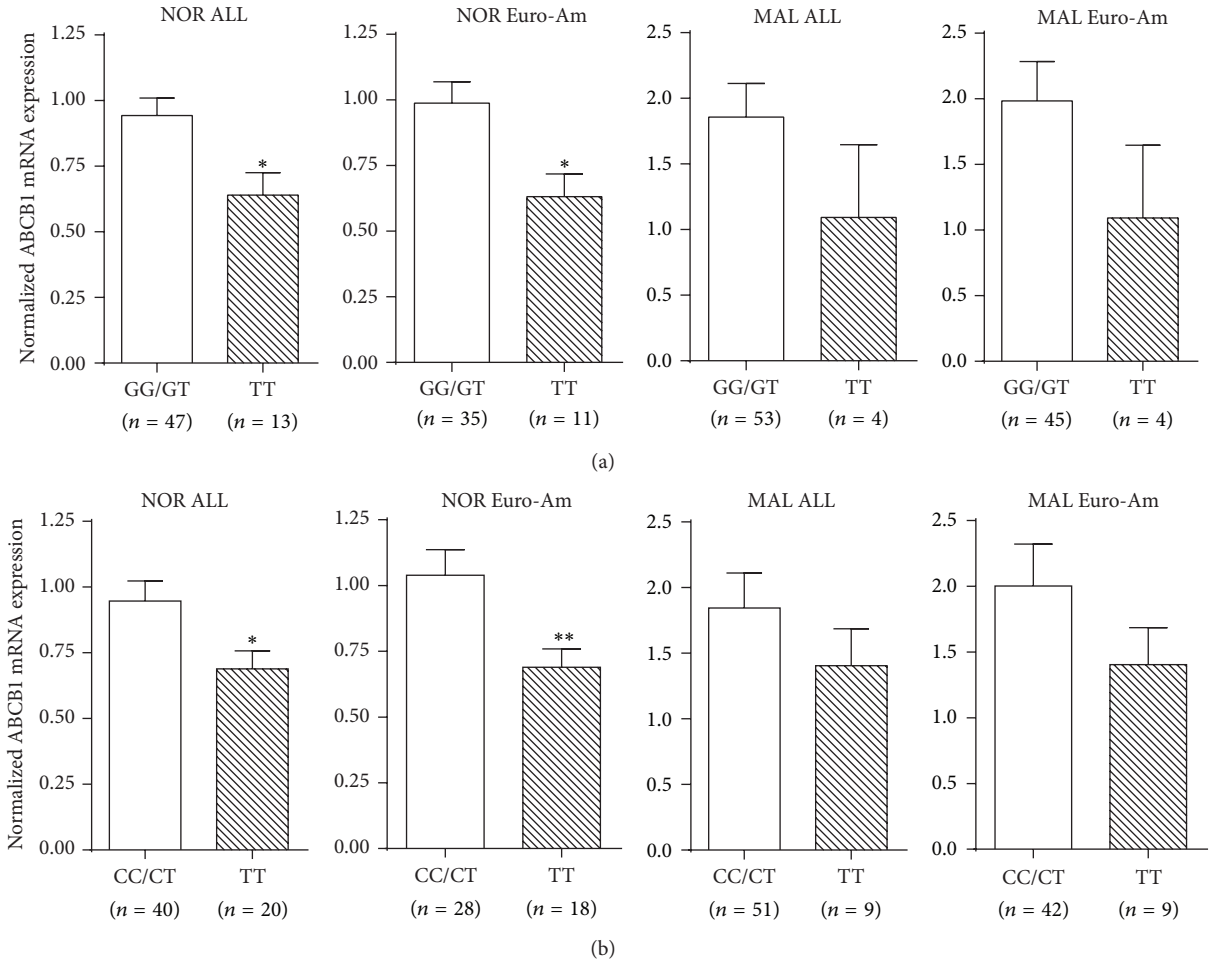

Figure 2: Expression of ABCB1 based on genotypes. The mRNA expression of ABCB1 in mutant homozygotes of G2677T (a) and C3435T (b) was compared to the wild-type homozygotes and heterozygotes in both whole study population (ALL) and the European Americans (Euro-Am). Values are expressed as means ± SEM, $^{*}P < 0.05$, and $^{**}P < 0.01$.

sample size, we could not test the association in African Americans. Even with additional samples, it would remain very difficult to obtain sufficient statistical power to detect the association because of the very low genotype frequencies of ABCB1 2677TT and 3435TT in African Americans [21–23]. To our knowledge, this is the first report of the common polymorphisms in the ABCB1 gene affecting the genetic risk of developing pancreatic cancer. More independent studies with larger sample sizes are needed to verify this interesting discovery.

ABCB1, ABCC1, and ABCG2 gene expression was significantly increased in the MAL specimens compared to NOR pancreases (Figure 1). This finding is consistent with other reports [9–12] for the potential involvement of ABC transporters in pancreatic cancer chemoresistance.

The ABCB1 2677TT and 3435TT genotypes/haplotypes were associated with reduced expression of ABCB1 in normal pancreatic tissue (Figures 2 and 3), although the differences were not statistically significant in pancreatic cancerous specimens, which might be because of the smaller sample size of TT variants in MAL group. In fact, this result is consistent with our observation that fewer carriers of ABCB1 2677TT and 3435TT developed pancreatic cancer (Tables 2 and 3). The nonsynonymous mutation in ABCB1 G2677T can result in a distinct amino acid change (Ala > Ser), which exhibited lower substrate specificity and reduced drug-stimulated ATPase activity as compared to the wild type [23]. The ABCB1 C3435T is a synonymous mutation but the variant can alter protein expression by affecting translation efficacy [24]. Since ABCB1 3435TT was first reported to be significantly associated with reduced ABCB1 expression in intestine compared to the CC homozygotes, numerous studies have investigated the association of ABCB1 genotype/haplotype with expression/function of the transporter in different tissues [25]. However, the results were not consistent. Our study is the first to compare the expression of ABC transporters in pancreatic tissue based on genotypes. Clearly, additional studies are necessary to confirm the results from this study.

Gemcitabine is commonly used as an adjuvant therapy for the treatment of postoperative pancreatic cancer and remains the standard of care for advanced pancreatic cancer, despite the very low patient response rate due to innate and acquired

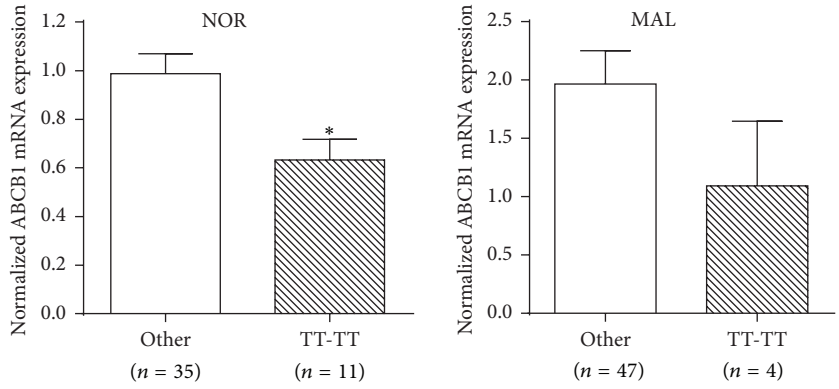

Figure 3: Expression of ABCB1 based on G2677T-C3435T haplotypes. The mRNA expression of ABCB1 in carriers of 2677TT-3435TT haplotype was compared to those of all the other haplotypes in European Americans. Values are expressed as means ± SEM and $^{*}P < 0.05$.

Table 4: Pancreatic cancer cell line ABCB1 mRNA expression, haplotype, and sensitivity to gemcitabine.

Cell line	ABCB1 expression	ABCB1 haplotype	GEM IC_{50} (μM)
AsPC-1	24.67	2677GG-3435CC	25.59
SU86.86	0.34	2677GG-3435CC	>100
PL-45	0.98	2677GG-3435CT	18.75
PANC-1	1	2677GT-3435TT	>100
BXPC-3	15.82	2677TT-3435TT	0.11
MiaPaca-2	0.75	2677TT-3435TT	1.90
CFPAC-1	1015.7	2677TT-3134TT	1.09

GEM: gemcitabine. The expression of ABCB1 gene is normalized to PMM1 and showed as x-fold relative to the expression in PANC-1 cells.

chemoresistance. While the human equilibrative transporters have been identified to be able to mediate gemcitabine uptake, the role of efflux pumps in gemcitabine transport has not yet been clearly delineated. Deoxycytidine kinase is the rate-limiting enzyme for gemcitabine activation and cytidine deaminase inactivates gemcitabine to difluorodeoxyuridine, allowing the metabolite to be cleared from the cell [26]. Interestingly, 2′,2′-difluorodeoxyuridine (dFdU), the major inactive metabolite of gemcitabine, is a substrate of ABC transporter [27]. Nonselectively inhibiting ABC transporters' activity could significantly increase intracellular dFdU level, inhibit cytidine deaminase, and result in an increase of intracellular gemcitabine concentration and enhanced cytotoxicity [27]. Although gemcitabine may not be a direct substrate of ABCB1, changes in expression or function of ABCB1 can be contributed to the development of gemcitabine chemoresistance. Due to high levels of ABCB1 gene expression being linked to poor prognosis of human pancreatic cancer [11] and the fact that ABCB1 2677TT and 3435TT genotypes were reported to be associated with increased overall survivals in gemcitabine treated postoperative pancreatic cancer patients [15], this study examined the association of ABCB1 genotype/haplotype with ABCB1 mRNA expression and the sensitivity to gemcitabine in several pancreatic cancer cell lines. Interestingly, we found that the cell lines with the ABCB1 2677TT-3435TT haplotype were more sensitive to gemcitabine than the cells carrying the other haplotypes (Table 4) but that the differences could not be explained by the varied expression of ABCB1 nor by the known mutations found in these cell lines [28]. The ABCB1 2677-3435 haplotypes in these pancreatic cancer cell lines might be associated with other unknown mechanisms that affect the cells' sensitivity to gemcitabine.

ABC transporters are not just drug efflux pumps. Many studies have elucidated the additional roles of ABC transporters in cancer initiation and progression [8]: for example, (1) ABCB1 has been reported to inhibit the apoptotic cascade in both normal and cancer cells; (2) knockdown of ABCB1 by small interfering RNA suppressed cancer cell proliferation and tumor expansion in a mouse xenograft model; (3) ABCB1 has also been reported to play a role in cell proliferation and delivering of protumorigenic platelet activating factor to its receptor. Any of these mechanisms could possibly explain the striking finding from this study that lower expression levels of ABCB1 in the normal 2677TT and 3435TT carriers are associated with reduced risk of developing pancreatic cancer.

ABCB1 G2677T and C3435T are two functional SNPs with varied frequencies in different populations [21, 22, 25]. The very low frequencies of 2677TT and 3435TT genotypes/haplotypes in African American populations may partially explain why African Americans are more frequently affected by pancreatic cancer than European Americans and Asian Americans [29]. African Americans have the highest incidence and mortality rates of pancreatic cancer compared to the other ethnic groups in the US [29]. The lack of protective genotype/haplotype, ABCB1 2677TT and

3435TT, may contribute to a higher susceptibility of African Americans to pancreatic cancer and increased likelihood of gemcitabine chemoresistance, thus poor prognosis.

In conclusion, this study has found that (1) the ABCB1 2677TT and 3435TT genotypes/haplotypes are associated with lower risk of developing pancreatic cancer; (2) the mRNA expression of ABCB1 reduced in the ABCB1 2677TT and 3435TT carriers; and (3) the ABCB1 2677TT-3455TT haplotype might be linked to an increased sensitivity to gemcitabine compared to the other haplotypes. The results from this study may aid in the future practice of utilizing pharmacogenomics to guide pancreatic cancer chemotherapy. However, large studies in different ethnic groups are needed to further confirm these findings.

Disclaimer

The views presented in this paper do not necessarily reflect those of the Food and Drug Administration.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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